INVESTIGATION OF AQUEOUS HUMOR FLOW IN THE RABBIT WITH THE USE OF FLUORESCEINATED DEXTRANS

SUSAN REDFIELD CARTER

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ABSTRACT

INVESTIGATION OF AQUEOUS HUMOR FLOW IN THE RABBIT

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In the study of aqueous humor dynamics in the eye, it is essential to have an accurate method of determining an absolute aqueous humor flow rate as well as acute and chronic changes in the rate. The purpose of this research was to design such a technique. Fluorescein isothiocyanate dextran (MW 156,900) was injected into the anterior chamber of the eyes of 13 pigmented rabbits. Fluorophotometry was then performed three hours after the injections to monitor fluorescein concentration in the anterior chamber over a period of three hours. Because the rabbit was awake, moving about and mixing the fluorescein dextran and fresh aqueous itself, the fluorescein concentration decreased according to first order kinetics, i.e. exponential decay. The linear plots of ln (fluorescein concentration) versus time also demonstrated first order kinetics. Aqueous humor flow rates were then calculated using the slope of the linear plots and the volume of the anterior chamber to obtain a mean flow rate of 2.79 $\pm$ 0.20 ul/min (N=13). An advantage to this new technique is that diffusional loss of fluorescein to the cornea and other ocular tissues is reduced because the fluorescein is bound to a large dextran molecule. Thus, this technique provides an accurate measurement of flow which can be used to further elucidate the mechanism of aqueous humor production, or to study new antiglaucomatous drugs.

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A Thesis Submitted to the Yale University
School of Medicine in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Medicine

by

Susan Redfield Carter

1989

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Marvin Sears MD, who has been an inspiration since my first year of medical school. Joseph Elman MD, Douglas Gregory PhD and Alden Mead deserve much thanks for their advice and encouragement. Thanks must also be given to Bob Brown for his photographic assistance. Finally, I would like to thank Didier for his constant words of support, and of course for his infinite computer wisdom!

This work was supported in part by a Fight For Sight Student Fellowship in honor of Elsie K. Sloate from Fight For Sight, Inc., New York City.

To my parents,

Priscilla and Richard

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INTRODUCTION

Aqueous humor is a fluid with many functions that is produced in the posterior chamber and flows through the anterior chamber of the eye. It supports the metabolism of the avascular lens and cornea, and contributes to the nutrition of the vitreous humor and the trabecular meshwork. Not only does it provide substrates, but it also carries away metabolic wastes from the eye. Most importantly, the formation of aqueous humor contributes to the maintenance of intraocular pressure.

The pathway of aqueous humor in the eye is the following. It is formed by both secretion of solute and osmosis of water from the epithelium of the ciliary body. The formation of the fluid is dependent mainly on the active transport of sodium by the non-pigmented epithelial cells of the ciliary processes that are closest to the posterior chamber (Cole, 1977). This creates an osmotic gradient, leading to the influx of water into the posterior chamber. From the posterior chamber, the aqueous humor flows through the pupil into the anterior chamber. The aqueous leaves the eye through the trabecular meshwork, passing into the canal of Schlemm. Via the collector channels in the sclera, the aqueous is carried to the episcleral veins, where it mixes with blood.

The two main factors concerned with the maintenance of intraocular pressure (IOP) are (1) the rate of aqueous humor production (F) and (2) the resistance to outflow (R) of the aqueous from the eye. The coefficient of the facility of outflow (C), or the ease with which the fluid exits, is the inverse of the resistance to outflow. Thus:

$$IOP \propto F \times R = F / C$$

The glaucomas are a group of ocular diseases characterized by an increase in intraocular pressure. This increased intraocular pressure may cause degeneration of the optic nerve with excavation of the optic disc, leading to visual field loss. Glaucoma is classified both by the configuration of the anterior chamber angle (open or closed), as well as by the apparent cause (primary, congenital or secondary). In open-angle glaucoma, the aqueous humor has free access to the outflow path through the trabecular meshwork because the angle between the cornea and iris is open. In closed-angle glaucoma, the root of the iris is in close apposition to the trabecular meshwork, preventing aqueous humor from leaving the eye. In primary glaucomas, the cause is unknown; in secondary glaucomas, the causes are many, some of which are inflammation, pigment dispersion, hemorrhage secondary to trauma, dislocated lens, ciliary body tumors, or corticosteroids.

The optic nerves have varying degrees of susceptibility to similar levels of intraocular pressure. There are certain people who demonstrate progressive disc cupping and field loss with normal or mildly elevated intraocular pressure. This entity is called low tension glaucoma, and may represent as much as 40% of all glaucomas.

Glaucoma is the second leading cause of blindness in the United States, with glaucoma blindness affecting approximately 17 per 100,000 persons. Over 11% of all blindness and 8% of visual impairment may be due to glaucoma (Leske, 1983). Primary open-angle glaucoma, the most common type, .represents 60% to 70% of all adult glaucoma. Although the aqueous humor has unimpeded access to the trabecular meshwork, there is abnormally high resistance to aqueous flow through the meshwork. The underlying abnormality in the trabecular angle tissue is currently being studied.

Because glaucoma progresses with an almost complete absence of symptoms until late in the course of the disease, and then presents with permanent visual field loss, early diagnosis is essential. The ophthalmologist should examine patients looking for elevated intraocular pressures, cupped optic discs, and gradual loss of peripheral vision. Unlike angle-closure glaucoma, primary open-angle glaucoma is treated medically, at first. Pharmacologic agents are used to lower the intraocular pressure. Thus, medications that are used to treat primary open-angle glaucoma either decrease the rate of aqueous humor production, increase the outflow of aqueous from the anterior chamber, or both. If the pressure cannot be controlled with medications, as indicated by persistently elevated intraocular pressures and by progression of optic nerve atrophy with associated visual field defects, laser trabeculoplasty or surgery may be indicated.

The study of aqueous humor dynamics, in particular the rate of aqueous humor flow, is crucial for furthering the understanding of the pathophysiology of elevated intraocular pressure and the mechanism of action of antiglaucomatous drugs. Aqueous humor flow can be defined as inflow, pupillary flow and outflow. Inflow describes the aqueous which is produced by the ciliary processes, pupillary flow that which passes through the pupil, and outflow that which passes through the chamber angle. These flow rates may differ slightly because of loss of aqueous humor from the posterior chamber to the vitreous or loss of aqueous from the anterior chamber to an alternate outflow path. For example, Bill (1966) showed the presence of uveoscleral routes for outflow in monkeys using labelled albumin and gamma globulin in a perfusion experiment. He was unable to demonstrate uveoscleral flow in rabbits using either red dextran (MW 40,000) or labelled albumin (Bill, 1966).

Methods previously developed to examine the rate of flow of aqueous humor may be classified into the following categories: (1) clearance of substances from the anterior

chamber (2) tracer dilution technique (3) anterior chamber perfusion (4) tonography and (5) photogrammetric determination. Each of these categories will now be discussed.

METHODS OF AQUEOUS HUMOR FLOW DETERMINATION

Clearance techniques

The rate of aqueous humor flow has been studied most extensively by researchers utilizing techniques that follow the clearance of a substance or substances from the anterior chamber. The majority of these methods determine the aqueous humor outflow. The most frequently used compound has been fluorescein, although para-amino hippuric acid, Rayopake, Diodrast, iodide, I-131 albumin and labelled hyaluronate have also been used.

Fluorescein is a yellow acid dye of the xanthene series with a molecular weight of 332 (Romanchuk, 1982). It is an excellent tracer to study the flow of aqueous humor for various reasons. Fluorescein is harmless to the eye, does not form bonds to vital tissues, can be detected noninvasively within the eye using a fluorophotometer, and does not alter the naturally existing state of intraocular pressure (Maurice, 1967). Also, the intrinsic efficiency of fluorescein in changing absorbed to emitted light is quite high.

Fluorescein is not an ideal substance in that it does bind reversibly to albumin, with unbound fluorescein in human plasma representing approximately 15% of the total (Brubaker, 1982). This binding partly quenches the fluorescence. The fluorescein also undergoes glucuronidation in the plasma, with the glucuronidation ratio reaching a maximum of 96% in 5 hours. If the fluorescein concentration is measured in the anterior chamber during this time, this changing level of fluorescence due to

glucuronidation must be taken into account. The fluorescence of fluorescein glucuronide is 1/53.8 of that of fluorescein (Araie, 1980). Although fluorescein does not bind tightly to tissues, it does rapidly penetrate the corneal endothelium from the anterior chamber. Relatively high concentrations of fluorescein in the cornea can interfere with measurements of low concentrations in the anterior chamber because of scatter (Brubaker, 1982). Despite these disadvantages, fluorescein remains an extremely useful substance in examining aqueous flow rates. It has been administered intravenously, orally, topically to the cornea via drops and iontophoresis, and intravitreally in an attempt to find an accurate method for measuring the flow rate.

Ehrlich, in 1882, was the first to use fluorescein in ophthalmologic research, to study the origin of aqueous humor. After administering fluorescein systemically, he observed it in the aqueous humor of the rabbit eye. It wasn't until the 1950's that Goldmann laid down the groundwork for further research of aqueous dynamics using fluorescein.

Goldmann (1950) examined the change in fluorescein concentration in the anterior chamber over time using a slit-lamp fluorophotometer. The fluorescein was given intravenously to human subjects. The slit-lamp fluorophotometer employed a subjective intensity matching technique. Goldmann derived the necessary theoretical concepts to then calculate the aqueous flow rate from the concentration of fluorescein in the anterior chamber and in the plasma. He also demonstrated that the transfer of fluorescein out of the anterior chamber has at least two components: bulk outflow through the angle and diffusion across the iris. Goldmann calculated the latter to be approximately one-tenth of the former. Although Goldmann's method required complex mathematical analysis, many blood samples, and the use of a subjective

fluorophotometer, it also demonstrated that aqueous flow could be determined without any interference in the eye except that of observation with a slit-lamp.

Langley and MacDonald (1952) were probably the first to administer fluorescein topically to the cornea in order to calculate aqueous humor flow rates using fluorophotometry. The corneal application technique became more accurate later on with the invention of improved fluorophotometers and the formulation of sound mathematical theories.

In 1954, Langham and Wybar designed the first objective slit-lamp fluorophotometer, which measured the fluorescence in the eye with a photomultiplier tube. Maurice made a significant contribution in 1963 with the development of a highly sensitive objective fluorophotometer that could measure fluorescent intensity in areas as small as the corneal stroma or as large as the entire cornea and anterior chamber. This instrument had an improved signal to noise ratio and an operator observable fluorescence window. With a modified version of this fluorophotometer, Jones and Maurice (1966) devised a method based on a valid theoretical model to measure aqueous humor flow using corneal application of fluorescein.

The method of Jones and Maurice (1966) involved the placement of a depot of fluorescein within the corneal stroma using iontophoresis, and observation of the fluorescence in the cornea and anterior chamber over time using fluorophotometry. The fluorescein gradually passed from the cornea into the anterior chamber. It was assumed that negligible amounts of fluorescein were lost from the cornea through limbal vessels. The fluorescein left the eye primarily by bulk flow with the aqueous humor through the chamber angle, although some also diffused into iris vessels, as proposed by Goldmann.

The concentration of fluorescein in the anterior chamber rose rapidly over the first two hours after iontophoresis, until a steady state was reached between the rate of passage from the cornea into the aqueous and the rate of loss of the dye from the aqueous through the trabecular meshwork. After that, the concentration of fluorescein in the aqueous fell, parallelling the rate of fall of fluorescein in the corneal stroma. When the measured corneal and aqueous concentrations of fluorescein were plotted against time on a semi-logarithmic scale, the falling phase was characterized by straight line graphs, illustrating the first order kinetics or exponential decay.

Jones and Maurice described two methods for determining the aqueous humor flow rate using this technique, each with a different mathematical basis. Method I examined the change in fluorescein concentration in the anterior chamber over time, measuring a transfer coefficient out of the anterior chamber. The volume of the anterior chamber must be measured separately to determine the absolute value for the flow rate. This method required measurements of the fluorescence in the anterior chamber during the early period following iontophoresis. During this time, not only was the eye subject to any side effects from the iontophoresis, but also the transfer of dye from cornea to anterior chamber was more irregular than during the falling phase of the concentration curve. Thus, measurements taken at this time may not have been as accurate as if they had been taken later.

Method II of Jones and Maurice involved the measurement of the ratio of total amount of fluorescence in the eye to that in the aqueous humor during the decaying phase of the concentration curve. This resulted in an absolute value for the aqueous humor flow rate of 2.5 $\pm$ 0.50 ul/min (N=10). This method was advantageous because it was a noninvasive method that could be used in humans to detect changes in aqueous humor flow within a given eye.

Various disadvantages existed for Method II. Determination of the total mass of fluorescein late in the procedure may have provided some experimental error, as the total amount became increasingly more difficult to measure once it was not in a relatively circumscribed area. Also, the conjunctiva hides about 10% of the anterior chamber and 20% of the cornea, which could falsely lower the total mass measured by 25%, thus lowering the apparent calculated flow rate (Coakes and Brubaker, 1979). If the assumption that fluorescein did not diffuse from the cornea into scleral vessels was incorrect, then the estimated flow rate would be falsely elevated. Despite these disadvantages, Method II of Jones and Maurice has been a frequently used method to calculate aqueous humor flow in humans since it was first described 22 years ago.

Starr (1966) used a variation of Method I of Jones and Maurice in humans with corneal iontophoresis of fluorescein to compare diurnal changes in the rate of flow in a given eye, as well as the response of the flow rate to intravenous acetazolamide. Bloom (1976) used the second method of Jones and Maurice with an objective slit-lamp fluorophotometer with improved electronic circuitry in humans, and obtained an average value of flow quite similar to that of Jones and Maurice.

Coakes and Brubaker (1979) described an alternate method of measuring aqueous flow based on that of Jones and Maurice, using corneal iontophoresis of fluorescein. Method I of Jones and Maurice required measurement of fluorescein concentration in the aqueous soon after iontophoresis, and Method II measurement of the total mass of fluorescein several hours after that. As discussed above, both of these measurements may be greater sources of error if taken at these times. Coakes and Brubaker's method was modified such that the aqueous fluorescence was determined later in the experiment when the concentration was changing less rapidly, and the total mass was measured early in the experiment when a more defined depot existed in the cornea.

Even with the use of modifications of Method II of Jones and Maurice and a nomographic analysis of the data, Coakes and Brubaker's values for flow remained in good agreement with those of Jones and Maurice. Brubaker (1982) examined another modification of the Jones and Maurice methods. Jones and Maurice's theories were based on a two compartment model of fluorescein kinetics, where the rate of loss of fluorescein from the cornea to the anterior chamber, and from the anterior chamber to the outflow path must be proportional to, respectively, the total mass of fluorescein in the cornea, and in the anterior chamber. This model assumed that fluorescein was not lost at the limbus and did not form concentration gradients within the corneal stroma. Brubaker's multiple compartment model divided the cornea into layers and concentric rings to simulate the movement of fluorescein within the stroma, at the limbus, and at the endothelial surface. Brubaker showed that there was not a clinically significant difference between the transfer coefficients calculated using the two different compartment models. This implied that limbal losses from the model cornea are negligible, and that the simple two compartment model of Jones and Maurice was sufficient for clinical studies.

In 1975, Nagataki described a method of measuring the transfer coefficients of fluorescein out of the anterior chamber due to both bulk flow (k_{fa}) and diffusion (k_{dpa}) after an intravenous injection of fluorescein. Jones and Maurice's method determined the total transfer coefficient (k_o) after fluorescein application to the cornea, where:

$$k_o = k_{fa} + k_{dpa}$$

Goldmann (1950) determined that the loss of fluorescein by diffusion is one tenth that by bulk flow. Elman (1986) calculated the diffusional loss of fluorescein to be 33%, by comparing the amount of fluorescein to the amount of blue dextran (MW 2,000,000)

remaining in the rabbit eye after intracameral injections of these two substances. Because of its molecular weight, it was assumed that blue dextran would leave the eye only be bulk flow. However, the percentage of diffusional loss may change in pathological processes or with administration of drugs. In fact, Elman (1986) determined that with larger flows the diffusional component appears to decrease. For this reason, Nagataki stressed the importance of being able to determine each coefficient separately.

Following an intravenous injection of fluorescein in humans, Nagataki measured the fluorescein concentration in serum samples, anterior chamber aqueous humor and pupillary aqueous humor using a fluorophotometer. The pupillary aqueous is that which passes through the pupil from the posterior to anterior chamber. Kinsey and Palm (1955) devised mathematical equations relating these various concentrations to the transfer coefficients of flow using radioactive sodium and thiocyanate, which Nagataki modified for his experiments.

Although Nagataki's method did give separate values for the transfer coefficients of flow, it also required fluorescence measurements in three different fluids, each contributing a source for error. Measurements of plasma and anterior chamber aqueous fluorescence were compounded by the fact that fluorescein binds to albumin, and that fluorescein undergoes glucuronidation in the plasma, a fact that was not taken into account in this method. Also, as pointed out by Nagataki, because fluorescein easily penetrates the endothelium of the cornea, the values determined for the diffusion transfer coefficient were probably underestimated. Determinations of pupillary aqueous fluorescence could be askew since the measurements were made through the fluorescent anterior chamber aqueous. Nagataki's method resulted in $k_{fa} = 0.922 \pm 0.073 \times 10^{-2}$, $k_{dpa} = 0.974 \pm 0.094 \times 10^{-3}$ and $k_o = 1.02 \pm 0.079 \times 10^{-2}$ min^{-1} (N=24). If the

anterior chamber volume is assumed to be 200 ul, the aqueous humor flow rate can be calculated by multiplying this volume by the transfer coefficient of bulk flow. Nagataki's flow rate is 1.8 ± 0.8 ul/min.

Araie (1980), working with Nagataki and Mishima, developed a method for measuring the transfer coefficients of bulk flow and diffusion using oral fluorescein. After an oral dose of fluorescein, the dye was rapidly absorbed, giving serum concentrations high enough so that the fluorescein passed into the anterior chamber and could be detected with fluorophotometry. The fluorescein concentrations in the serum and aqueous humor rose more slowly than those following an intravenous injection of fluorescein. Araie believed that the rapid rise of the concentrations in the serum and aqueous after an intravenous dose could cause an amplification of the transfer coefficient; therefore, oral administration was preferable.

However, many more variables were introduced with this method than with the intravenous technique. The fluorescence of the plasma could be affected by the rate of absorption of fluorescein from the gastrointestinal tract, the rate of its excretion by the kidneys, the degree of binding to albumin, and the level of glucuronidation of the fluorescein. Similar to intravenous fluorescein, oral fluorescein would enter the cornea both from the limbal vessels into the peripheral cornea and from the anterior chamber into the stroma. Araie and Maurice (1985) showed that the corneal endothelial permeability differs for fluorescein and for fluorescein glucuronide, which is a larger molecule and has a lower lipid solubility than fluorescein. This would have an effect on the fluorescein concentration within the anterior chamber. Thus, although the oral fluorescein method provided separate values for the transfer coefficients and may have been safer than the intravenous route, it was not a straightforward method.

In addition to topical, intravenous and oral routes of administration, fluorescein has also been given intravitreally in the form of fluoresceinated dextrans. Johnson and Maurice (1984) injected fluorescein dextran into the vitreous humor of rabbits. The fluorescein dextran diffused forward into the anterior chamber at a relatively steady rate and flowed out the chamber angle in the aqueous humor. The concentration of the fluorescein dextran in the vitreous and the aqueous was monitored with fluorophotometry over a period of weeks, with the concentration in the aqueous peaking one to six days after the intravitreal injection. The concentrations in the aqueous and vitreous fell in parallel, in an exponential fashion. If the aqueous flow rate increased, the concentration of fluorescein dextran in the anterior chamber decreased. The absolute flow rate could be calculated from the total amount of dextran in the eye, and its concentration in the anterior chamber.

The intravitreal fluorescein dextran method was advantageous in that flow could be followed continuously in one animal for a period of weeks. Thus, long term effects of drug administration or dosing regimens could be examined in one animal. Also, the technique of monitoring the concentrations was noninvasive, after the initial intraocular injection was made. The major problem with this method was the difficulty in determining the total amount of fluorescein dextran left in the eye at the end of the experiment, which was used to calculate the absolute flow rate. Johnson and Maurice did not publish an absolute flow rate for this reason. Araie (1985) using this method obtained a mean aqueous flow rate in 20 rabbits of 3.64 ± 0.15 ul/min. Another disadvantage was that acute changes in flow secondary to drugs could not be detected because of the slow turnover of the aqueous in the anterior chamber.

Other investigators have worked with a variety of other substances besides fluorescein to determine the aqueous flow rate using the general principle of clearance of

substances from the anterior chamber. Barany and Kinsey (1949) worked out a method of measuring aqueous flow in rabbits using substances intravenously that could be rapidly cleared from the blood by renal excretion after they entered the anterior chamber. The substances used were para-amino hippuric acid (PAHA), Rayopake and Diodrast. When the plasma concentration of the substance was extremely low because of efficient renal excretion, the rate of loss of the substance from the anterior chamber was nearly the same as the flow rate. The rate of decay of the substance in the anterior chamber was then determined by removing samples of aqueous, first from one eye and then the other, at different time periods and comparing the concentration of the compound in each sample. The plasma concentration of the substance was also measured at the same time points to provide a correction for any additional substance that may have entered the anterior chamber from the blood. The resulting flow rate calculated by Barany and Kinsey (1949), assuming an anterior chamber volume of 250 ul, was 2.80 ul/min using PAHA, 2.75 ul/min using Rayopake and 2.55 ul/min using Diodrast.

Besides being an invasive method, this technique had the disadvantage of using data from two sets of rabbits to determine the flow rate. Because a small amount of the test substance was in the blood during the measurement of the aqueous concentrations, a factor based on the steady state distribution ratio of the substance in the plasma and aqueous was utilized to correct for this. The steady state data was collected from a group of rabbits different from those whose flow rates were being measured. This could have caused an error in the determination of the flow rate in an individual rabbit.

Barany and Wirth (1953) resolved this problem by calculating both the flow rate and the steady state ratio in the same animal. Rabbits were given infusions of PAHA to maintain constant plasma levels. To obtain the steady state ratio, plasma and aqueous samples were taken just before the infusion was stopped. The experiment then proceeded

like that of Barany and Kinsey, with an aqueous sample being taken from the other eye at a later time, resulting in a calculated flow rate of 2.50 ul/min. However, the eyes cannot always be assumed to be absolutely identical. Also, it is frequently desireable to maintain one eye as a control, and examine the other eye under varying experimental conditions.

Linner (1953) proposed the following solution to this issue. Several test substances, preferably isotopic, could be administered to the animal at different times with suitable intervening intervals. One sample of aqueous humor would then determine the concentration of different test substances at different points in time, thus generating several points on a time concentration curve.

Becker (1962) did just that by administering several different isotopes of iodide intravenously and intraperitoneally at different times prior to tapping the anterior chamber. Although the flow rate could be determined in a single eye, changes in the flow rate could not.because only one invasive tap of the anterior chamber could be made at a specific time.

Both Maurice (1959) and O'Rourke and Macri (1970) used noninvasive external monitors to measure the clearance of radioactive isotopes from the anterior chamber of rabbits. Maurice's experiment was an important predecessor of the noninvasive fluorescent studies. Maurice injected radioactive iodide-labelled albumin intravitreally in rabbits and then monitored the decrease in total activity over time with a scintillation counter over the eye. From this information, the transfer coefficient of flow out of the vitreous could be calculated. The transfer coefficient of outflow from the anterior chamber was determined from samples of the aqueous and vitreous collected by paracentesis at the end of the experiment. The resulting transfer coefficient was lower

than most determined in rabbits. A possible explanation for this is that either the radioactive albumin was not leaving the vitreous by way of the anterior chamber or that the outflow channels were partially blocked secondary to some inflammation observed by Maurice.

Maurice's method was crucial in that it illustrated a noninvasive way of monitoring a substance withih the eye. However, it still had the disadvantage of giving only the flow rate at the time of the termination of the experiment. O'Rourke and Macri (1970), eleven years later, also used radioactive albumin with an external monitor to measure flow. However, their experiments were performed in humans using microinjections of the tracers into the anterior chamber. Because the tracer was initially confined to the anterior chamber, the additional variable of transfer of albumin from the vitreous to aqueous in Maurice's experiment was eliminated; however, there still may have been diffusional loss of albumin to other ocular tissues.

Laurent and Fraser (1983) also injected a tracer directly into the anterior chamber to measure aqueous flow in anesthetized rabbits. Sodium hyaluronates of varying molecular weight were injected intracamerally via a needle placed in the anterior chamber by a needle gun. The needle gun was designed by Sears (1960) to reduce inflammation (Sears, 1960). At the termination of the experiment, the aqueous humor sample was withdrawn and radioactivity in the sample measured. The results were expressed in terms of the fraction of injected material remaining in the aqueous after a given time. The method is comparable to that of Becker except for the route of administration of the tracer and that the rabbits must be under general anesthesia. Barbiturate anesthesia reduces the flow rate of aqueous humor in rabbits (Becker, Krupin, Podos, 1970), which may explain why Laurent and Fraser's value for the rate constant was lower than that in studies in which general anesthesia was not used.

Tracer dilution techniques

Tracer dilution techniques differ from clearance methods in that they measure the dilution of the tracer by the newly produced aqueous humor, not the disappearance of the tracer from the anterior chamber. Thus, tracer dilution methods determine the inflow. In 1967, Oppelt measured aqueous humor formation rates in anesthetized cats using labelled inulin as a tracer. Two cannulas were introduced into the eye: one in the posterior chamber for infusion of an inulin containing aqueous humor-like buffer, and the second in the anterior chamber near the iridocorneal angle for collection of the aqueous. The infusion rate was adjusted to maintain a constant intraocular pressure. The aqueous flow rate was calculated knowing the rate of the infusion and the dilution of the labelled inulin as it passed through the two chambers.

With this method, the flow rate could be determined continuously in one eye, which made the technique ideal for examining changes in flow following drug administration. However, it also required serious alterations in the normal physiology of the eye. The cats were under general anesthesia, which as discussed can have an effect on flow rate. Two cannulas had to be inserted into the eye, which might initiate an inflammatory response mediated by prostaglandins (Beitch and Eakins, 1969) (Podos, 1973). The inflammatory response is characterized by miosis, hyperemia of the conjunctiva and iris, breakdown of the blood aqueous barrier, and transient elevation of the intraocular pressure (Neufeld, 1972). Thus, the aqueous humor formation rate could be increased as a result of intraocular inflammation. Finally, the infusion itself caused an alteration in the intraocular pressure indicating a corresponding artificially induced change in flow.

In 1979, Green used an inulin perfusion technique in rabbits similar to that of Oppelt, except the infusate was directed into the anterior chamber instead of the posterior chamber. The experimental setup was felt to be unstable with the inlet needle in the posterior chamber. High perfusion rates were used to ensure adequate mixing of the perfusate with the newly formed aqueous. The calculated aqueous flow rate was 1.62 ± 0.08 ul/min (N=8). This method, like Oppelt's, was performed under general anesthesia in association with significant invasive procedures.

Miichi (1982) determined the aqueous humor formation rate in anesthetized rabbits using fluoresceinated dextrans in a method similar to that of Oppelt. The posterior to anterior chamber perfusion set up was like Oppelt's except that the rabbits were pretreated with indomethacin to reduce an inflammatory response. Pretreatment of rabbits with intraperitoneal injections of indomethacin, an inhibitor of prostaglandin synthesis, has been shown to blunt the intraocular inflammatory response to trauma (Sears and Sears, 1974). Aspirin, another prostaglandin inhibitor, has also been shown to decrease any inflammatory reaction to ocular trauma when rabbits were given 600 mg per rectum one hour prior to ocular procedure (Neufeld, 1972) (Podos, 1973). Although a potential inflammatory response had been dampened, the technique was still performed on animals under general anesthesia.

Perfusion

The problem of invasive techniques and the need for general anesthesia was also an issue for the perfusion method. This method was similar to that of the tracer dilution technique in that cannulation of the anterior chamber was required. Becker and Constant described a method of perfusion in 1956 where saline was infused into the anterior chamber, causing an elevation in the intraocular pressure. The aqueous outflow could be

calculated by multiplying the constant ratio between the infusion rate and the resultant pressure change by the difference between the normal intraocular pressure and the episcleral venous pressure. Becker and Constant calculated a flow rate of 3.5 ul/min using this technique. One problem that they encountered was spontaneous or induced rises in intraocular pressure during the experiment, due possibly to deep anesthesia, trauma secondary to the cannulation, sudden alteration of the infusion pressure or prolonged perfusion.

Sears (1960) introduced an improved method of perfusion by using a mechanical needle gun to insert the cannula into the eye. The inflammatory response in the eye that can be induced by mechanical irritation of the cornea, conjunctiva, iris and lens could be reduced by using the needle gun. The inflammatory reaction, as discussed above, is accompanied by an increase in intraocular pressure. Sears observed that spontaneous changes in the intraocular pressure were minimized by inserting the cannula using the needle gun. Thus, this technique was valuable for lengthy studies of aqueous flow in one animal. Sears used a constant low infusion rate to determine flow at steady state. The aqueous flow rate determined by Sears was 2.1 ± 0.12 (N=10) and by Sears and Barany (1960) was 1.9 ± 0.22 (N=55).

Although many researchers performing perfusion experiments reported that the increase in intraocular pressure was directly proportional to the infusion rate, Langham (1959) noted some nonlinearity of the intraocular pressure. He showed that the increase in intraocular pressure actually caused a decrease in either the rate of aqueous humor formation or an increase in the resistance to outflow of aqueous. The clinical counterpart of this is pseudofacility, defined as the pressure sensitive part of aqueous humor formation (Brubaker and Kupfer, 1966). Since this is a negligible fraction, it does not receive the attention now that it did initially. However, because of

the alteration of the normal aqueous dynamics by both creating an elevated intraocular pressure with the infusion and using general anesthesia, the perfusion method may not have been determining an accurate flow rate.

Tonography

Tonography, another method for determining aqueous outflow rates, was also affected by pseudofacility. Tonography as it is used today was first described by Grant in humans (1950). The method involved the placement of a tonometer on the cornea for a period of four to six minutes. During this time, the intraocular pressure would at first rise because of the weight of the tonometer on the eye, and then fall as the weight of the tonometer caused an increased outflow of aqueous humor. The fluid displaced from the eye when the tonometer was placed on it is directly related to the increase in intraocular pressure and the facility of outflow of the aqueous humor, when volume corrections are applied. Friedenwald (1948) devised tables relating the intraocular pressure and corneal coefficient of rigidity to the volume of tonometric indentation for human eyes. The coefficient of outflow facility could then be calculated using the tonometer readings and corresponding displacement volume from the table. From this coefficient and the steady state intraocular pressure, the rate of aqueous flow under steady state conditions could be determined.

Several assumptions exist in this method, one of which was that Friedenwald's tables calculated for pressure and volume relations were accurate. Kornbluth and Linner (1955) determined that the tables could be used with reasonable accuracy in rabbits because the ocular rigidity of rabbit eyes is fairly close to that of humans. However, the flow rates determined by tonography depend on variables in addition to the ocular rigidity, such as the size of the eye, corneal curvature and episcleral venous

pressure. Kornbluth and Linner (1955) determined the aqueous flow rate to be 4.37 ± 1.87 (N=14) in rabbits under general anesthesia.

The most important assumption made was that an increase in the intraocular pressure of 10 - 15 mm Hg did not significantly affect the aqueous humor formation or outflow resistance. As discussed above, Langham (1959) showed that this assumption was not true. Thus, the outflow facility measured by tonography was actually shown to be the sum of the pseudofacility and the true outflow facility of the conventional outflow channels. Kupfer and Ross (1971) devised a method for calculating pseudofacility.

Because tonography required the placement of a tonometer on the eye for a period of four to six minutes, the first experiments done in rabbits were performed under general anesthesia (Kornbluth and Linner, 1955) (Linner and Prijot, 1958) (Prijot and Stone, 1958). Becker and Constant (1955) and later Lieb (1957) showed that tonography could be performed in rabbits with topical anesthesia if they were wrapped tightly in a sheet. Although general anesthesia reduces the intraocular pressure (Stone and Prijot, 1955), the flow rates determined under general anesthesia were not lower than those determined with topical anesthesia. This may be because the method itself gave such varying results from experimenter to experimenter (see Appendix, Table 2).

Photogrammetric technique

The last method of determining aqueous flow to discuss is the photogrammetric technique, one which differs significantly from the others discussed so far. Holm (1968) and Holm and Wiebert (1968) devised a method to measure the pupillary flow, or that aqueous passing from the posterior to the anterior chamber in humans. The anterior chamber was stained with fluorescein via corneal iotophoresis of fluorescein.

The pupil was made miotic with pilocarpine. The pupillary flow could be observed as a growing vesicle of non-stained aqueous coming through the pupil from the posterior chamber. An assumption was made that the non-stained aqueous flowing out of the pupil was the total amount of aqueous entering or leaving the anterior chamber at the time of the observation. Thus, the rate of growth of the vesicle equaled the rate of flow of aqueous through the pupil. The vesicle volume was determined photogrammetrically. Photographs of the vesicle were taken in rapid succession and the volume of the vesicle was calculated since the distances between successive sections is known. Five to fifteen seconds later, a new series of photographs was taken of the slightly enlarged vesicle. The flow rate, 3.1 ± 1.6 ul/min (N=17), was equal to the change in vesicle volume divided by the time between volume measurements.

Krakau (1969) modified this technique with a new method for measuring the volume of the aqueous vesicle. Instead of photographing the vesicle, Krakau used a photomultiplier tube to measure the fluorescence of a region enclosing the pupil. The newly formed vesicle, with its non-fluorescent aqueous, caused a fall in the intensity of the fluorescence as it grew in size. Pupillary flow was equal to the change in intensity over time multiplied by a correction factor.

Despite the fact that this was the most direct method for calculating flow, the photogrammetric analysis of the vesicle volume was quite complicated. In addition, any small movement of the head of the subject could disturb the photographic recording of the vesicle size. The most significant problem with this method was that the pupil must be miotic to visualize the vesicle and pilocarpine must be administered. Pilocarpine has been reported to both increase the outflow facility and decrease aqueous formation in humans (Barsam, 1972). Thus, the pupillary flow rate being measured might be

reduced because of pilocarpine administration. Also use of this method to examine the effects of other drugs on flow could be complicated by the presence of pilocarpine.

Current Research

The methods for determining aqueous humor flow discussed above all have various advantages and disadvantages to them. In the study of aqueous humor dynamics in the eye, the method used is very important. (See Appendix for tables summarizing flow rate data for humans and rabbits.) The technique should give an accurate repeatable measurement of flow that is as direct as possible. The procedure should be simple and noninvasive during the actual determination of flow., and must not alter the normal physiology of the eye. Thus, the method should not require general anesthesia. Finally, the technique should be able to determine acute and chronic changes in the flow rate as well as an absolute value for aqueous flow.

There is much to be accomplished with an accurate method for determining aqueous flow rates. The mechanism of production of aqueous humor could be further elucidated. New antiglaucomatous agents could be tested for their efficacy and their mechanism of action determined. Flow rates in different glaucomatous states could be analyzed.

A method to measure flow that addresses some of the above requirements was created. The method developed involves injections of fluoresceinated dextrans into the anterior chamber of rabbit eyes, followed by fluorophotometry to measure the decrease in the fluorescein dextran concentration over time. The hypothesis of this research was that this method would provide an accurate direct simple determination of aqueous humor flow in rabbit eyes. This was shown by the resultant small error of the mean

flow rate calculated and the use of this method to calculate acute changes in the flow rate. Acetazolamide, because of its ability to acutely decrease aqueous inflow, was used to demonstrate the effectiveness of this method in determining such acute changes in aqueous flow.

MATERIALS AND METHODS

FLUORESCEIN DEXTRAN

The fluorescein isothiocyanate dextran (FITC dextran) obtained from Sigma Chemical Company (St. Louis, MO), has an average molecular weight of 156,900 and a Stokes's radius of 8.7 nm. Phosphate buffer was prepared, with pH=7.6, the pH of rabbit aqueous humor (Kinsey and Reddy, 1964). 200 mg of fluorescein isothiocyanate dextran was dissolved in 10 ml of buffer to obtain a stock solution with a concentration of 2×10^7 ng/ml.

RABBITS

Dutch Belted male pigmented rabbits were used in all experiments. They weighed between 1.5 and 2.5 kg at the time of the study.

ANESTHESIA

Both general and topical anesthesia were required for the intracameral injections of fluorescein dextran. The rabbits were injected intramuscularly with a 1:1 mixture of ketamine hydrochloride (Vetalar - Parke-Davis, Syracuse, NY) 25 mg/kg and xylazine (Rompun - Miles Laboratories, Shawnee, KS) 25 mg/kg one and a half hours prior to the intracameral injection procedure. One drop of 0.5% proparacaine hydrochloride (Ophthetic - Allergan, Irvine, CA) was applied immediately prior to the intracameral injection. Neither general nor topical anesthesia was needed when the measurements of fluorescein concentration in the eye were taken.

PRETREATMENT

Rabbits were pretreated with aspirin, a prostaglandin synthesis inhibitor, to minimize any inflammatory response which might have an effect on the aqueous humor flow rate. A 600 mg aspirin suppository was inserted rectally into each rabbit, after general anesthesia had taken effect.

INTRACAMERAL INJECTION

The intracameral injections were made one hour after the rabbits were pretreated with aspirin. The rabbit was positioned with its head tilted to give sufficient exposure of its right eye, but without exerting pressure on the left. 10 ul of 2 x 10^7 ng/ml fluorescein isothiocyanate dextran were drawn up into a 30 gauge Hamilton syringe. The needle was wiped dry and 2 ul of air was drawn up to avoid the staining of the cornea as the needle passed through it. One drop of Ophthetic was applied to each eye of the rabbit before the eye was proptosed with two soft cotton applicator sticks.

The intracameral injection was made with the use of a Zeiss surgical microscope. The needle penetrated the corneal epethelium at the periphery of the cornea, with the needle parallel to the limbus. A several millimeter tract was made within the corneal stroma before pushing the needle into the anterior chamber. The eye was released from its proptosed position immediately before the injection. The fluorescein dextran was injected away from the point of entrance into the chamber. The needle was quickly withdrawn from the eye and no leakage of fluorescein dextran was seen from the self sealing tract. The other eye was then injected using the same procedure.

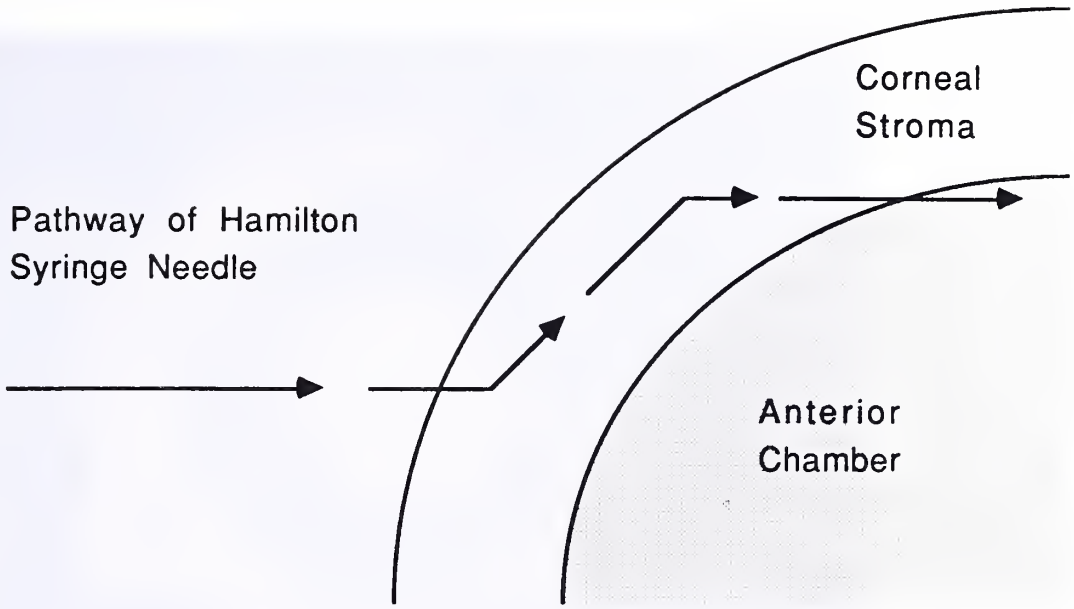


Figure 1: Pathway of Hamilton syringe needle through the cornea to the anterior chamber for injection of fluorescein dextran.

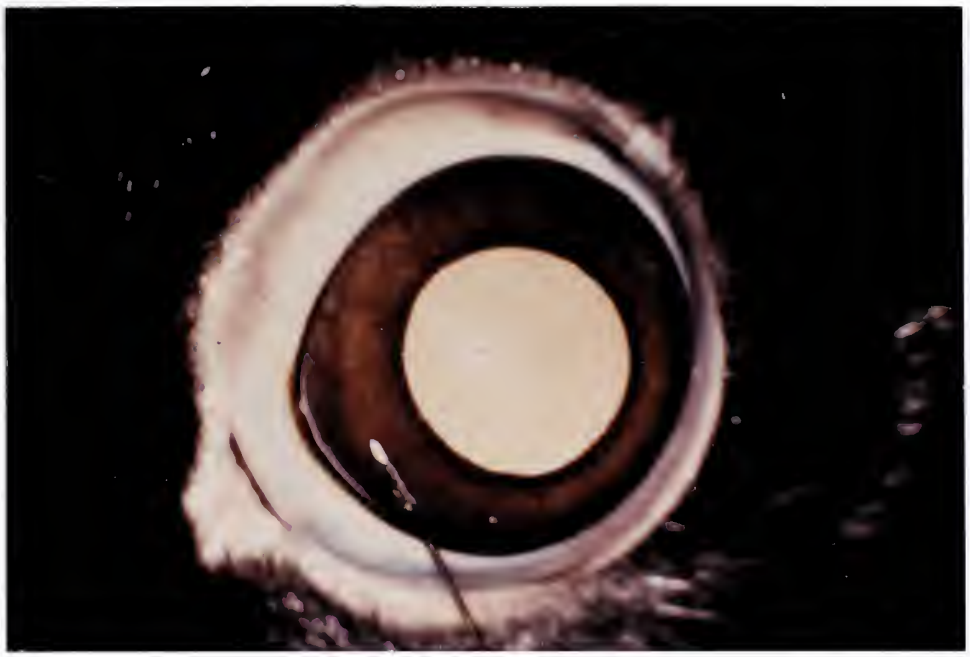


Figure 2: Advancement of Hamilton syringe needle within corneal stroma.

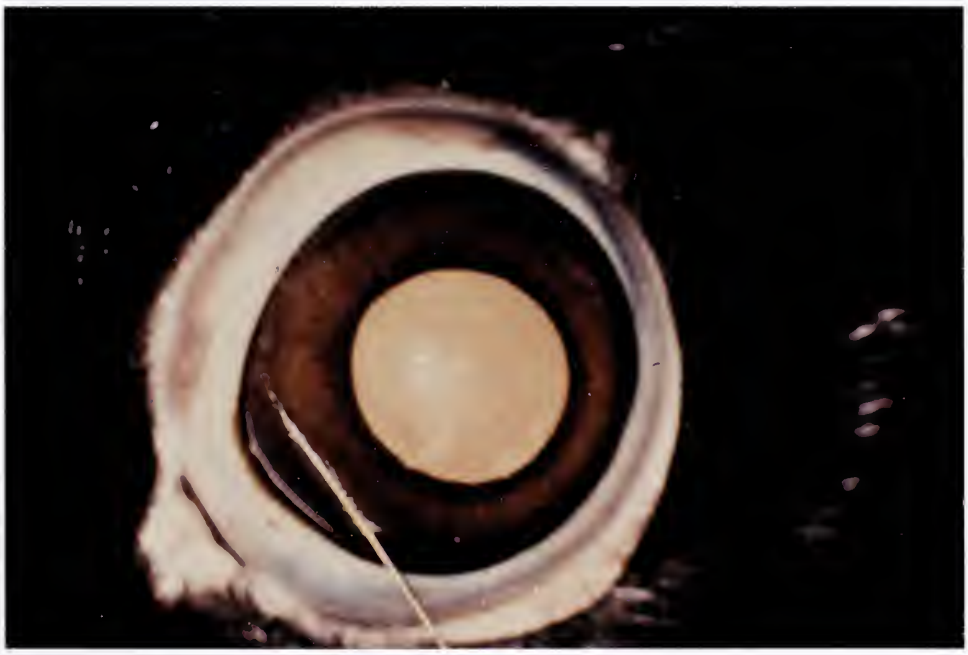


Figure 3: Entrance of needle tip into the anterior chamber.

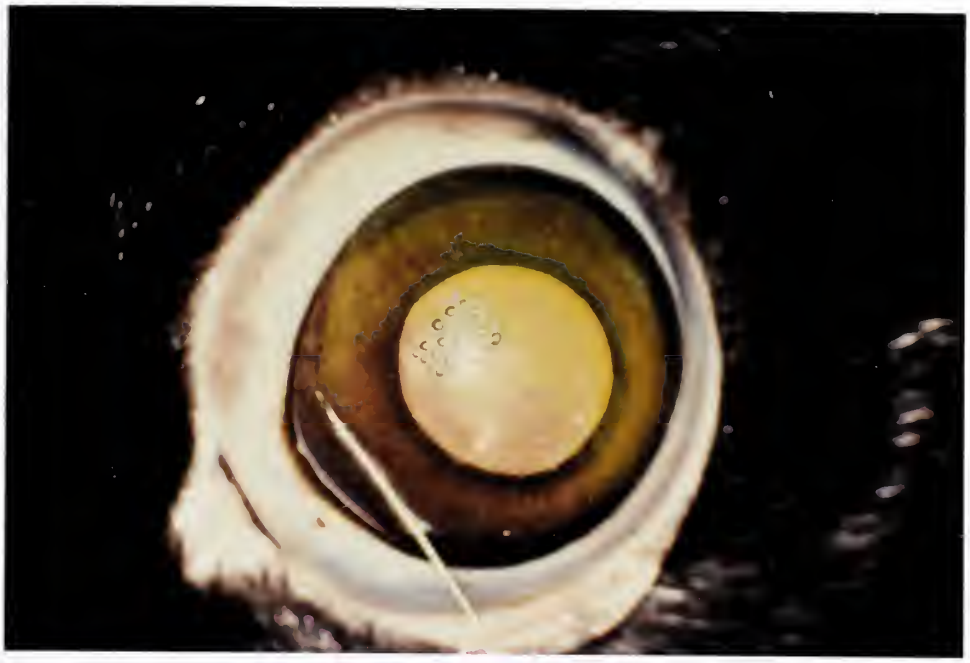


Figure 4: Injection of 10 ul of fluorescein dextran into anterior chamber.

Figure 5: Final appearance of eye following injection and thorough mixing of fluorescein dextran with aqueous humor.

With the rabbit still anesthetized, its head was gently shaken for ten minutes to provide initial mixing of the fluorescein dextran with the aqueous humor. Three hours following the intracameral injection, the rabbit was awake, moving about and mixing the fluorescein dextran and fresh aqueous itself. Fluorophotometry was performed on the rabbit at this time.

FLUOROPHOTOMETRY

The Coherent Fluorotron is a scanning fluorophotometer which determines the concentration of fluorescein at different positions anteriorly to posteriorly within the eye. Thus, the cornea, anterior chamber and lens are scanned consectively. Each scan is composed of single readings at 148 positions which are 0.5 mm apart. Each reading takes 25 msec resulting in a total scan time of 9 seconds. A sample scan is below.

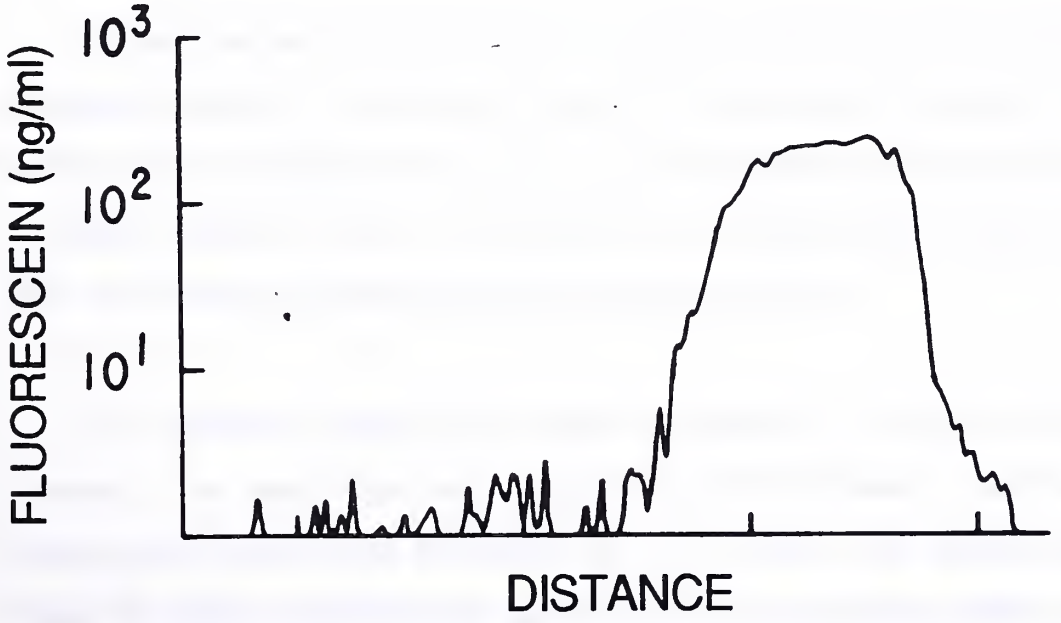


Figure 6: Sample Fluorotron scan of rabbit eye.

The average concentration of fluorescein in the aqueous humor can be calculated by the Fluorotron using a calibration factor for fluorescein dextran, and the span of positions over which the concentration was measured specifically in the anterior chamber. The calibration factor is determined by comparing known fluorescein dextran concentrations to the fluorescein concentration determined by the Fluorotron scan. From a stock solution of flurescein dextran, concentrations ranging from 0 to 100,000 ng/ml were made in buffered solution at pH=7.6 and then put in quartz cuvettes to be scanned by the Fluorotron. When the Fluorotron scan concentration data was plotted against the fluorescein dextran concentration, the method of least squares and a linear regression formula was used to determine the slope (4.66×10^{-3}), the y-intercept (1.77) and the correlation coefficient of 0.9999 for 17 points (see Appendix, Figure 1). Thus, the calibration formula is the following:

$$\text{Actual FITC Dextran Conc (ng/ml)} = (\text{Fluor scan (ng/ml)} - 1.77) / 4.66 \times 10^{-3}$$

Fluorophotometry was performed on the awake rabbits three hours following the intracameral injections. This allowed enough time for the rabbits to completely recover from the general anesthesia, as well as for the concentration of fluorescein dextran in the anterior chamber to fall to a concentration on the linear portion of the standard curve. The scans were always performed at the same time of day.

The rabbit was strapped into a holder and placed on a table in front of the Fluorotron. The rabbit's head was positioned such that the beam of light from the fluorophotometer would enter the rabbit's eye in the center of the cornea and pass through the pupil. If necessary, the rabbit's eye lids were held open without exerting pressure on the globe. The scan was commenced by activating the Fluorotron with a foot

pedal. This procedure was repeated for each eye every 20 to 30 minutes for approximately three and one half hours.

In two animals, an intravenous injection of acetazolamide (Diamox) 25 mg/kg was given two and a half hours after scanning was commenced. It is known that acetazolamide reduces flow rates by 40% to 60% ,with its effect being evident one to four hours after intravenous administration (Macri, 1975). Fluorophotometry was continued during this time period to examine the effectiveness of this method in detecting changes in the flow rate.

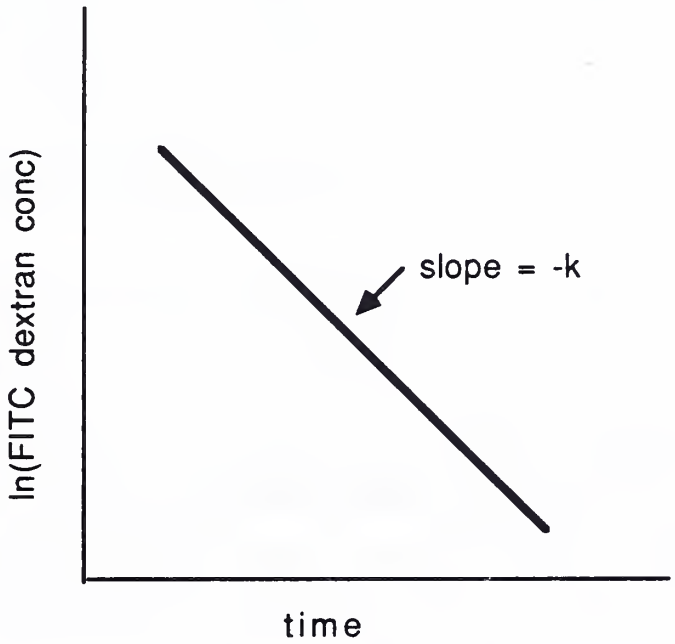
MATHEMATICAL ANALYSIS

An assumption is made that the fluorescein dextran concentration in the anterior chamber is decreasing according to first order kinetics, or exponential decay, as it is diluted by and thoroughly mixed with the newly produced aqueous humor. Any outflow of aqueous will not affect the concentration of fluorescein dextran in the eye, because the aqueous humor that leaves the eye has the same concentration of fluorescein dextran as in the rest of the anterior chamber. Thus, the aqueous humor inflow rate is being measured with this technique. The flow rate of aqueous humor can be calculated as the fractional decrease in fluorescein dextran concentration per minute multiplied by the volume of the anterior chamber.

$$\text{Flow} = (\ln(c_1) - \ln(c_2))\ V\ /\ (t_1 - t_2)$$

where c_1 = calibrated concentration of fluorescein dextran in anterior chamber at time t_1, c_2 = calibrated concentration of fluorescein dextran in anterior chamber at time t_2, t = time and V = volume of anterior chamber.

From the data obtained with fluorophotometry, the log of the fluorescein dextran concentration is plotted against time. The slope of the straight line graph is determined using the method of least squares with a linear regression analysis, and the flow rate is calculated by multiplying the negative value of this number by the volume of the anterior chamber, assumed to be 250 ul (Kinsey and Reddy, 1964) (see Appendix, Figures 2 - 14).



$$\text{Slope} = (\ln(c_2) - \ln(c_1)) / (t_2 - t_1) = -k$$

$$\text{Flow} = k\ V$$

For the rabbits that were given acetazolamide, a flow rate was calculated for the two hours prior to the administration of the drug, and for the period one to three hours after the administration. The two flow rates were compared to determine a percentage change in flow (see Appendix, Figures 15,16).

RESULTS

Intracameral injections and fluorophotometry were performed in thirteen pigmented rabbits. No adverse responses were noted to the injection, with a maximum of 0.5 ul leakage immediately after withdrawal of the needle and no leakage of fluorescein dextran from the injection site noted during the experiment. The corneal injection sites remained slightly edematous throughout the procedure. Small bubbles of air that appeared in the anterior chamber following the injection, as a result of drawing air up into the syringe to avoid staining the cornea, aided in the mixing of the fluorescein dextran and aqueous and were rapidly absorbed.

The rabbits tolerated the fluorophotometry well. The rabbit was immobilized by using a holder with velcro straps over the front and back portions of its body, leaving its head and legs free. The scan was repeated if the rabbit moved its head or eye during the nine second scan. If there was a technical problem with the injection, or if the curve did not fit a first order exponential decay, this data was not included in the final determination of the flow rate. For example, during the injection of three different eyes, the rabbit moved its head. The resulting data from these eyes was discarded. Also in three eyes, the ln(fluorescein dextran concentration) versus time graph did not result in a straight line, possibly due to inadequate mixing of the fluorescein dextran with the aqueous humor. For each rabbit the flow rate for the right eye was averaged with that from the left. The mean values for each rabbit were then averaged to obtain a final aqueous humor inflow rate of 2.79 ± 0.20 ul/min (mean ± SEM, N=13).

RABBIT #	RIGHT EYE	LEFT EYE	MEAN FLOW
1	3.72		3.72
2	2.48		2.48
3	1.49	1.83	1.66
4		2.74	2.74
5	3.57	4.20	3.89
6	3.19	3.38	3.29
7	2.23	2.03	2.13
8	1.89		1.89
9	2.36	2.43	2.40
10		3.05	3.05
11	3.20	2.88	3.04
12	4.08	3.21	3.65
13	2.35		2.35

MEAN FLOW RATE = 2.79 ± 0.20 ul/min (N=13)

Table 1: Summary of flow rate data.

For the two rabbits treated with acetazolamide, flow rates were calculated for time periods before and after administration of the drug. The percentage reduction in the aqueous humor flow rate was calculated for the four eyes and the values ranged from 37% to 58% with the average being 47%.

DISCUSSION

To evaluate the effectiveness of this method in measuring the aqueous humor flow rate, the various advantageous components of the procedure as well as the fulfillment of the criteria discussed in the introduction will be examined.

FLUORESCEIN DEXTRAN

The use of fluorescein isothiocyanate dextran to measure flow rates is beneficial for several reasons. Not only does the tracer fluoresce allowing external noninvasive monitoring of the decrease in concentration, but it is also a large molecule which will not diffuse back into the cornea or other ocular tissues as easily as fluorescein would. As seen by the Fluorotron scans of eyes in this experiment, there was no appreciable fluorescence in the cornea. Thus, this method will give a more accurate measure of bulk flow than those methods which used just fluorescein, since the diffusion component to flow is minimized. Despite its large size (156.900 MW, Stokes's radius of 8.7 nm), the fluorescein dextran flows out of the anterior chamber through the normal outflow channels without difficulty. Particles up to 800 nm can penetrate the aqueous outflow tract (Huggert, 1955).

ANESTHESIA

General anesthesia can reduce the rate of aqueous production, as discussed earlier. Although this method does require the rabbits to undergo general anesthesia, it is only during the intracameral injection and not when the actual measurements of the fluorescein concentration are being made. Four hours pass between the time of induction of anesthesia and the commencement of fluorophotometry. The anesthesia wears off in

approximately three hours, and all animals were active at the time scanning was begun. Thus, the general anesthesia probably does not have an effect on flow, but does enable an intracameral injection to be made.

INTRACAMERAL INJECTION

The technique for the fluorescein dextran intracameral injection involved a self-sealing needle tract within the corneal stroma. This is advantageous because leakage is reduced, with no more than 0.5 ul leaking out immediately after withdrawal of the syringe. Although this may reduce the total amount of fluorescein in the anterior chamber, it will not affect the rate constant used to calculate flow as long as no additional leakage occurs.

The intracameral injection enables the flow to be measured as directly as possible, because the fluorescein is deposited directly into the aqueous humor. The substance does not have to diffuse through the cornea or plasma to reach the anterior chamber, as in other methods. To minimize any inflammatory response created by the injection, the animals are pretreated with aspirin. Although the technique does require an invasive procedure, it occurs at the beginning of the experiment and does not disturb the normal physiology of the eye during the actual flow measurements.

FLUOROPHOTOMETRY

The scanning fluorophotometer used in this experiment has an advantage over the earlier models used in fluorescein flow experiments. It determines the fluorescein concentration for the anterior chamber by taking an average of the concentrations

measured at 0.5 mm intervals within the chamber. The earlier models detected the concentration at just one point.

MATHEMATICAL ANALYSIS

The relatively simple data analysis makes this technique quite appealing. The mathematical analysis is based on the assumption that the fluorescein concentration in the anterior chamber decreases exponentially, by first order kinetics, as it is mixed with the newly produced aqueous humor. This principle is validated by the straight line graphs that resulted from the semi-log graph of the concentration versus the time in 20 eyes. The linear regressions performed on this data give correlation coefficients with an average value of 0.975. Nonlinear graphs occurred for three eyes.

MEASUREMENTS OF FLOW

It is now important to discuss the results of this technique and whether this method fulfills the advantageous requirements discussed earlier. The flow rate calculated using the proposed method gives a value with a small standard error of the mean, demonstrating the precision of the technique. The actual accuracy of the method cannot be evaluated because the true value of the flow rate is not known. This method is attempting to determine the true value. However, the flow rate from this method is in good agreement with those previously published using different techniques (see table).

The method does not alter the normal intraocular aqueous dynamics during the period when the fluorescein concentration measurements are taken. The rabbits are pretreated with aspirin to prevent breakdown of the blood - aqueous barrier following the injection. As a result, no evidence of an irritative response from the injection, such

as miosis or iris hyperemia, is detected. The intraocular pressure increases a few millimeters of mercury just after the 10 ul anterior chamber injection, but returns to normal within five minutes (Gregory et.al., 1981b). The presence of fluorescein dextran does not impede flow in any way because the dextran is of a small enough radius to pass through the trabecular meshwork. The technique used to make the intracameral measurements,fluorophotometry, is noninvasive. Both general anesthesia and an invasive injection are required earlier in the experiment, but as discussed above these do not affect the actual flow determinations.

Not only does this method result in an absolute value for inflow, but it also can be used to determine acute changes in flow after the administration of a drug. Following an intravenous dose of acetazolamide in two rabbits, an appropriate 47% reduction in aqueous production occurred. Thus, the method described in this thesis fulfills the criteria for an effective method for determining the aqueous humor flow rate.

Potential shortcomings of this method must be evaluated. If leakage were to occur from the injection site during the course of the experiment, the flow rate would be artificially elevated, but only if the leakage were significant enough to cause a large change in anterior chamber volume. With the injection technique used in the study, no loss of aqueous or fluorescein dextran was observed during the experimental period. Loss of fluorescein dextran to other ocular tissues may occur, but as shown by Cole and Monro (1976) is minimal. Cole and Monro perfused the eyes of rabbits with a 0.5% fluorescein dextran solution (MW 150,000) at 54 ul/min continuously for 90 minutes, and examined the postmortem distribution of the fluorescein dextran in the ocular tissues. The amount of fluorescein dextran remaining in the cornea, and the iris and ciliary body, expressed as percentages of the total amount of fluorescein dextran lost from the perfusate during the perfusion period was 0.28% and 1.27% respectively.

If there was inadequate mixing of the fluorescein dextran with the aqueous at any point during the procedure, the fluorescein concentrations measured would have been erratic and could have given rise to either too high or too low a flow rate. The initial mixing of the fluorescein dextran with the aqueous was performed by gently shaking the rabbit's head for ten minutes.

Elman (1986) examined the effectiveness of this action. After induction of general anesthesia and pretreatment with a 600 mg aspirin suppository, a 10 ul injection of fluorescein was made into the anterior chamber. The rabbits heads were shaken for ten minutes. Then, simlutaneous paracentesis of the anterior chamber with two 27 gauge needles was performed to obtain a small and a large sample of aqueous humor. A statistically significant difference did not exist between the fluorescein concentrations in the two different sized samples. Thus, shaking the rabbit's head following the fluorescein injection provides adequate mixing.

During the course of fluorophotometry the rabbits were awake and active, mixing the newly formed aqueous with the previously mixed aqueous and fluorescein dextran. This is confirmed by the actual fluorophotometry data. The scans determined the fluorescein concentration at 0.5 mm positions from front to back of the eye. The scans, graphed as the fluorescein concentration versus distance, showed elevated concentrations of fluorescein for the entire depth of the anterior chamber, represented by an elevated horizontal line. In addition, if the mixing was insufficient, then the semi-log graphs of fluorescein concentration versus time would not have been straight lines. Linear graphs did result in 20 out of 23 eyes.

Although the method of intracameral fluorescein dextran does determine a production rate of aqueous humor in the eye, it may not be measuring the total amount of

aqueous humor formed by the ciliary processes. If some aqueous humor diffuses backwards into the vitreous, then this method is actually measuring that aqueous which is flowing into the anterior chamber, or the pupillary flow.

A final potential disadvantage to this method is that it should not be used in humans with good vision to determine aqueous humor flow rates because of the initial invasive injection. However, it can be used in many species of animals as well as consenting humans with blind eyes prior to enucleation of the eyes.

FUTURE RESEARCH

Various applications exist for this effective method of measuring aqueous humor flow rates in rabbits. The technique could be used to further elucidate the mechanism of aqueous humor production. Gregory et al. (1981a) have demonstrated the presence of a beta-adrenergic receptor in the ciliary process of the rabbit eye with a direct ligand-binding assay using ^{125}I hydroxybenzylpindolol. When cholera toxin, an irreversible activator of adenylate cyclase, was administered intra-arterially to rabbits, the flow rate of aqueous humor was decreased by 50% (Gregory, 1981b). Sears (1981) concluded that the beta-adrenergic receptor of the ciliary process is linked to the process of aqueous humor formation and may regulate the rate of aqueous formation. Stimulation of the receptor would cause a decrease in the rate of aqueous production.

The direct role of the beta-adrenergic receptor in aqueous dynamics can be further studied using the fluorescein dextran method described above. By administering isoproterenol, one can determine the effect of a beta-adrenergic agonist on the aqueous flow rate. A better understanding of aqueous dynamics may help comprehend the

pathophysiology of glaucoma, as some instances of glaucoma may be the result of receptor-type defects.

Finally, the method can be used to develop and test new pharmacologic agents for the treatment of glaucoma, as well as study the mechanism of action of known antiglaucomatous drugs. With early detection and treatment of glaucoma with useful drugs, the debilitating loss of vision can hopefully be slowed.

APPENDIX

AUTHOR	YEAR	METHOD	n	FLOW (ul/min)
Goldmann	1950	IV Fluorescein	10	1.9 ± 0.4
Goldmann	1951	IV Fluorescein	36	2.2 ± 0.4
Nagataki	1975	IV Fluorescein	24	1.8 ± 0.8*
Jones + Maurice	1966	Iontoph Fluor Method 2	10	2.5 ± 0.5
Starr	1966	Iontophoresis Fluor	8	1.9
Bloom	1976	Iontophoresis Fluor	19	2.8 ± 0.6
Yablonski	1978	Iontoph Fluor Method 2	15	2.3 ± 0.7*
Coakes + Brubaker	1978	Iontoph Fluor Method 2	20	2.7 ± 1.0
Coakes + Brubaker	1978	Iontoph Fluor Nomogram	20	2.9 ± 0.4
Brubaker	1981	Iontophoresis Fluor	113	2.4 ± 0.6
Araie	1980	Oral Fluorescein	10	1.8 ± 0.4*
Grant	1950	Tonography	34	3.66
Kupfer + Ross	1971	Tonography	4	1.09 ± 0.086
Holm	1968	Photogrammetric Method	17	3.1 ± 1.6
Holm + Wiebert	1968	Photogrammetric Method	17	3.4 ± 1.7

Table 1: Summary of Flow Rate Data for Humans

* = Flow rate calculated from rate constant assuming anterior chamber volume of 200 ul.

AUTHOR	YEAR	METHOD	n	FLOW (ul/min)
Barany + Kinsey	1949	IV PAHA	23	2.8*
Barany + Kinsey	1949	IV Rayopake	27	2.75*
Barany + Kinsey	1949	IV Diodrast	18	2.55*
Barany + Wirth	1953	IV PAHA	13	2.50*
Becker	1962	IV Iodide	15	3.75*
Kinsey + Reddy	1964	Na24 + Cl36	10	3.30
Maurice	1959	Intravitreal Albumin	7	2.13*
Araie	1985	Intravitreal Fl. Dextran	20	3.64 ± 0.15
Green	1979	Inulin Perfusion	8	1.62 ± 0.08
Miichi	1982	Fluor Dextran Perfusion	10	3.56 ± 0.40
Laurent + Fraser	1982	Intracameral Hyaluron.	20	2.35 ± 0.12*
Carter	1989	Intracameral Fl. Dextran	13	2.79 ± 0.20
Becker + Constant	1956	Perfusion	28	3.5
Langham	1959	Perfusion		2.4
Sears	1960	Perfusion	10	2.1 ± 0.12
Sears + Barany	1960	Perfusion	55	1.9 ± 0.22
Kornbluth + Linner	1955	Tonography	14	4.4 ± 1.87
Becker + Constant	1956	Tonography	28	3.5
Lieb	1957	Tonography	12	3.7
Prijot + Stone	1958	Tonography	16	3.2 ± 0.27
Prijot + Stone	1958	Tonography	22	3.7 ± 0.25
Linner + Prijot	1958	Tonography	14	2.84
Linner + Wistrand	1963	Tonography	7	5.18 ± 1.13

Table 2: Summary of Flow Rate Data for Rabbits

* = Flow rate calculated from rate constant assuming anterior chamber volume of 250 ul.

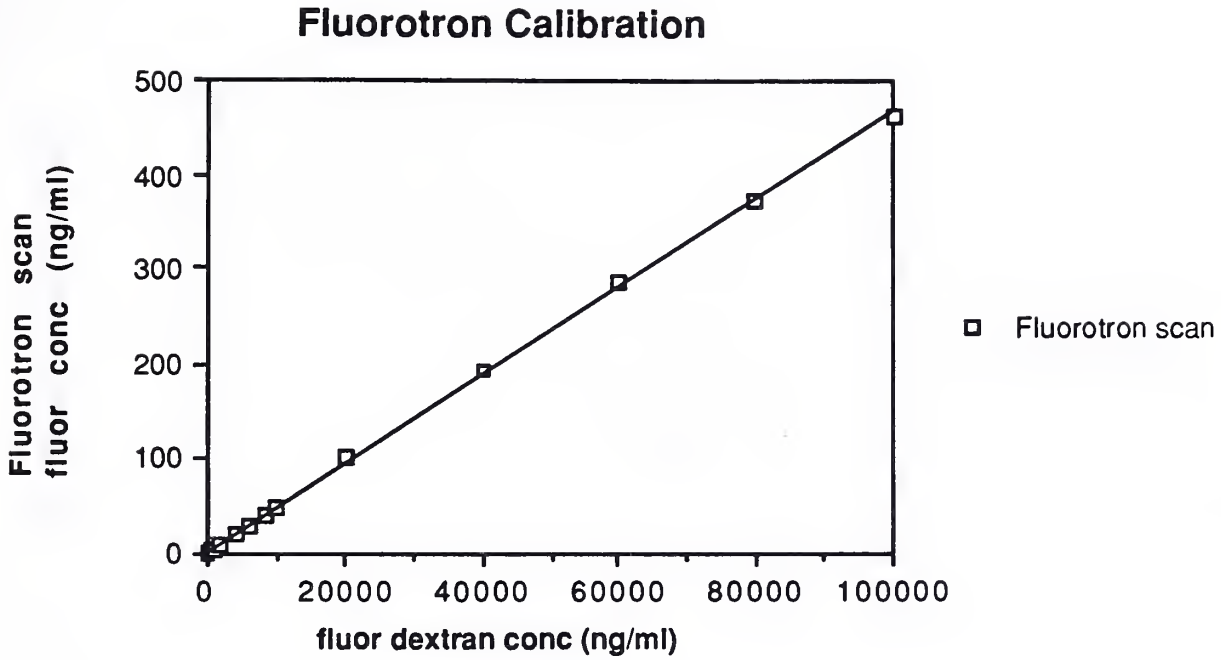


Figure 1: Determination of calibration factor for Fluorotron with various fluorescein dextran concentrations at pH=6.

Slope	4.66×10^{-3}
Y - Inter.	1.77
Corr. coeff.	0.9999

Actual Fluorescein Concentration = (scan - 1.77) / 4.66×10^{-3}

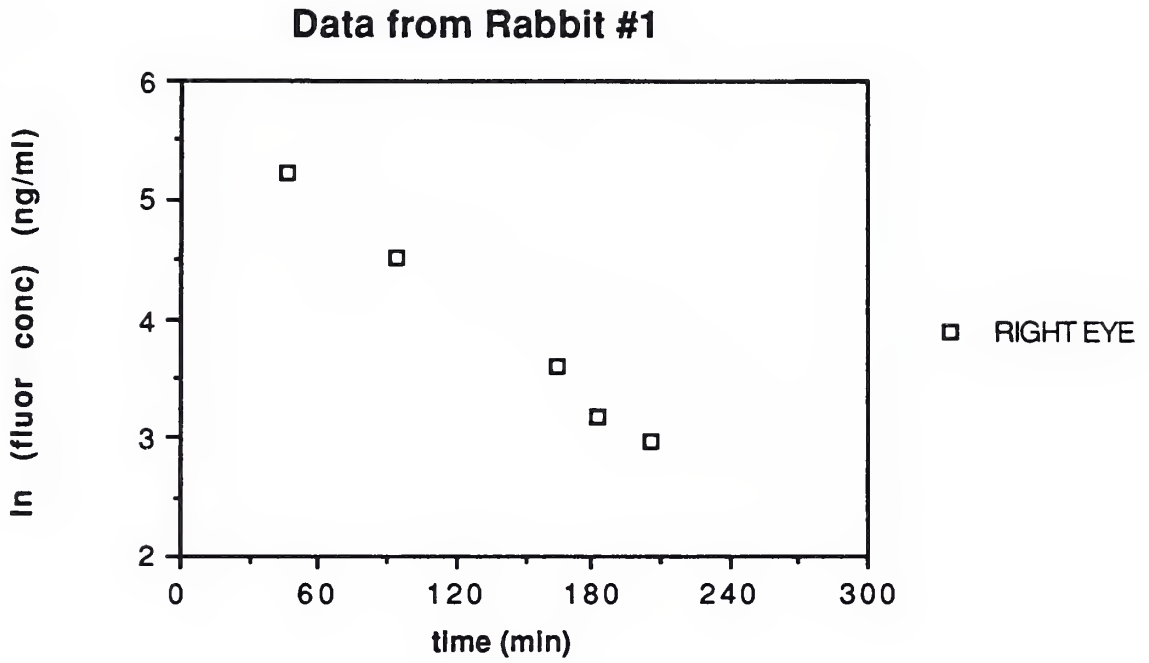


Figure 2: Aqueous humor flow determination for Rabbit #1. Scanning was begun 2 hours 45 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE
Flow (ul/min)	3.72
Slope	-0.015
Corr. coeff.	0.998

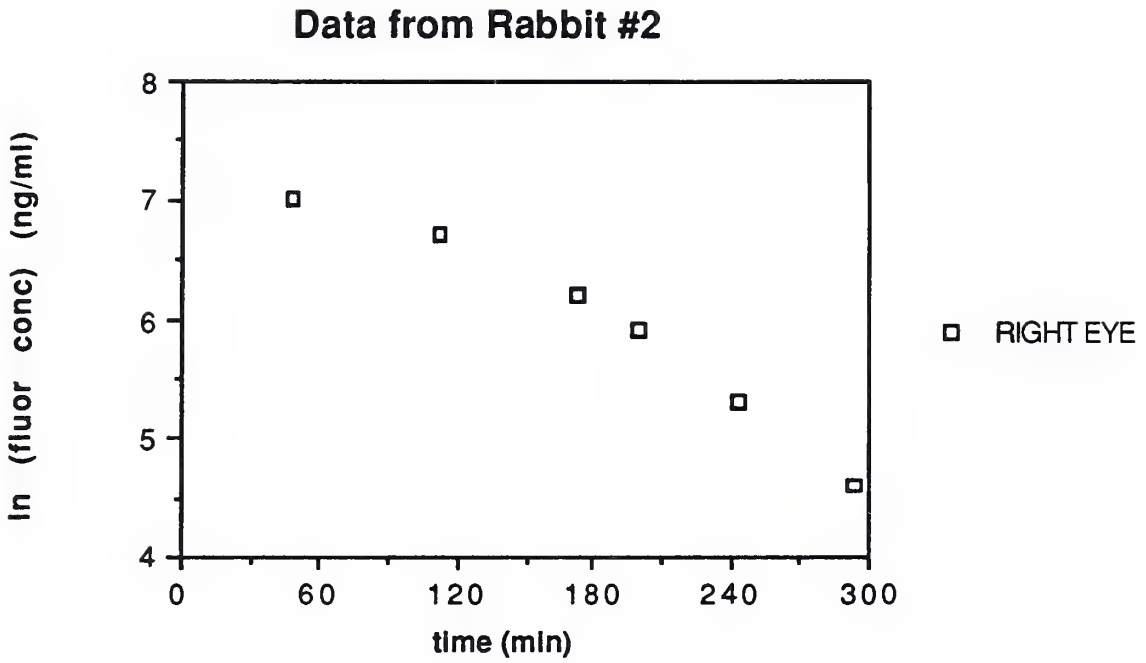


Figure 3: Aqueous humor flow determination for Rabbit #2. Scanning was begun 2 hours 30 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE
Flow (ul/min)	2.48
Slope	-9.93×10^{-3}
Corr. coeff.	0.98

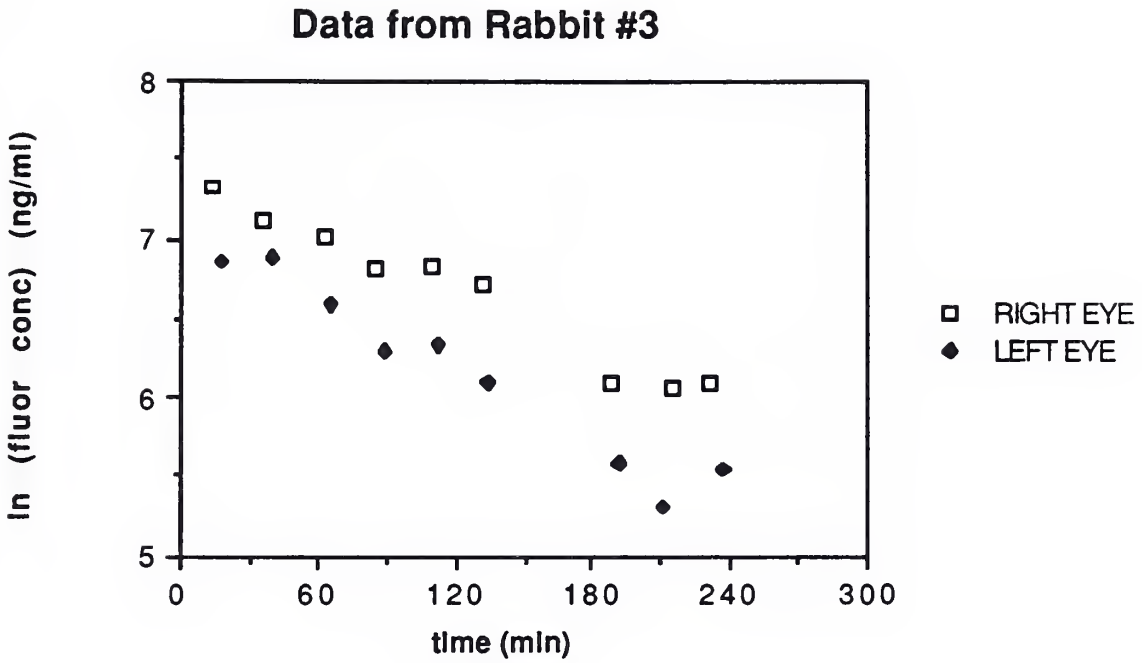


Figure 4: Aqueous humor flow determination for Rabbit #3. Scanning was begun 2 hours 35 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE	LEFT EYE
Flow (ul/min)	1.49	1.83
Slope	-5.97×10^{-3}	-7.32×10^{-3}
Corr. coeff.	0.97	0.97

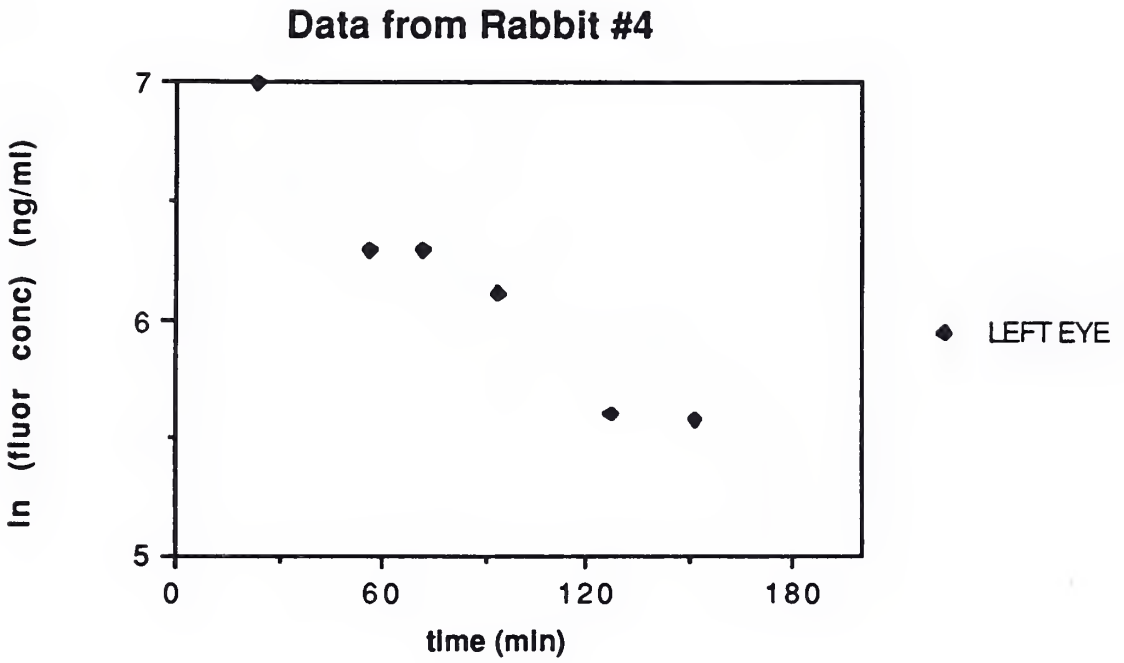


Figure 5: Aqueous humor flow determination for Rabbit #4. Scanning was begun 2 hours 30 minutes after 10 ul intracameral injection of fluorescein dextran.

	LEFT EYE
Flow (ul/min)	2.74
Slope	-0.11
Corr. coeff.	0.97

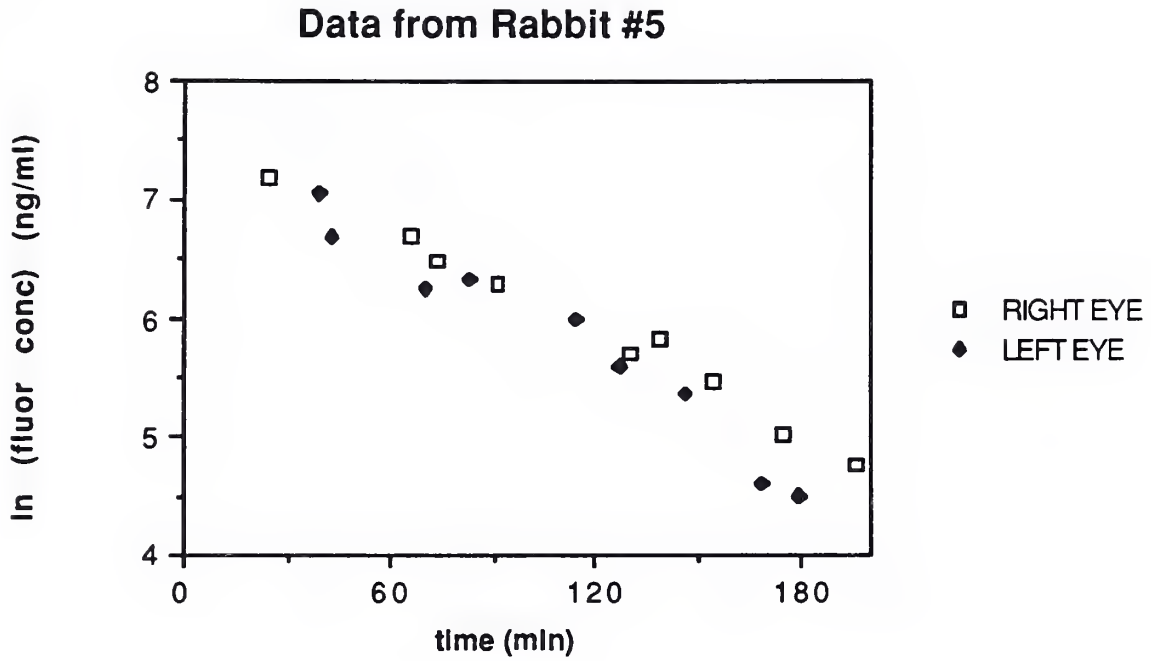


Figure 6: Aqueous humor flow determination for Rabbit #5. Scanning was begun 3 hours 10 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE	LEFT EYE
Flow (ul/min)	3.57	4.20
Slope	-0.0143	-0.0168
Corr. coeff.	0.993	0.98

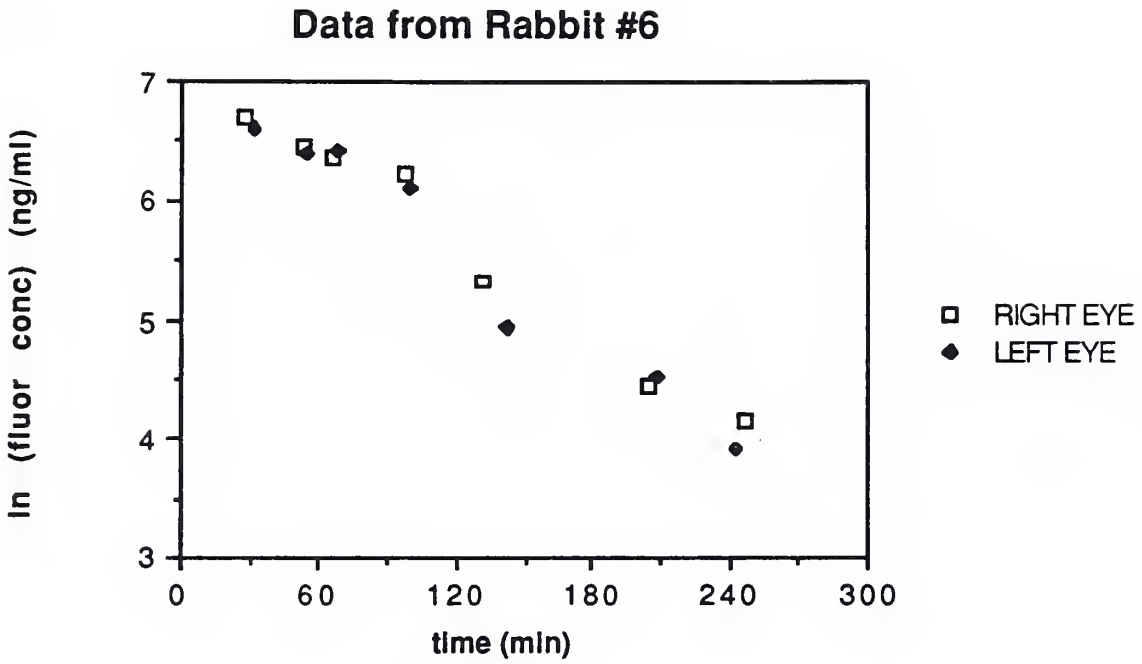


Figure 7: Aqueous humor flow determination for Rabbit #6. Scanning was begun 2 hours 30 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE	LEFT EYE
Flow (ul/min)	3.19	3.38
Slope	-0.0128	-0.0139
Corr. coeff.	0.986	0.981

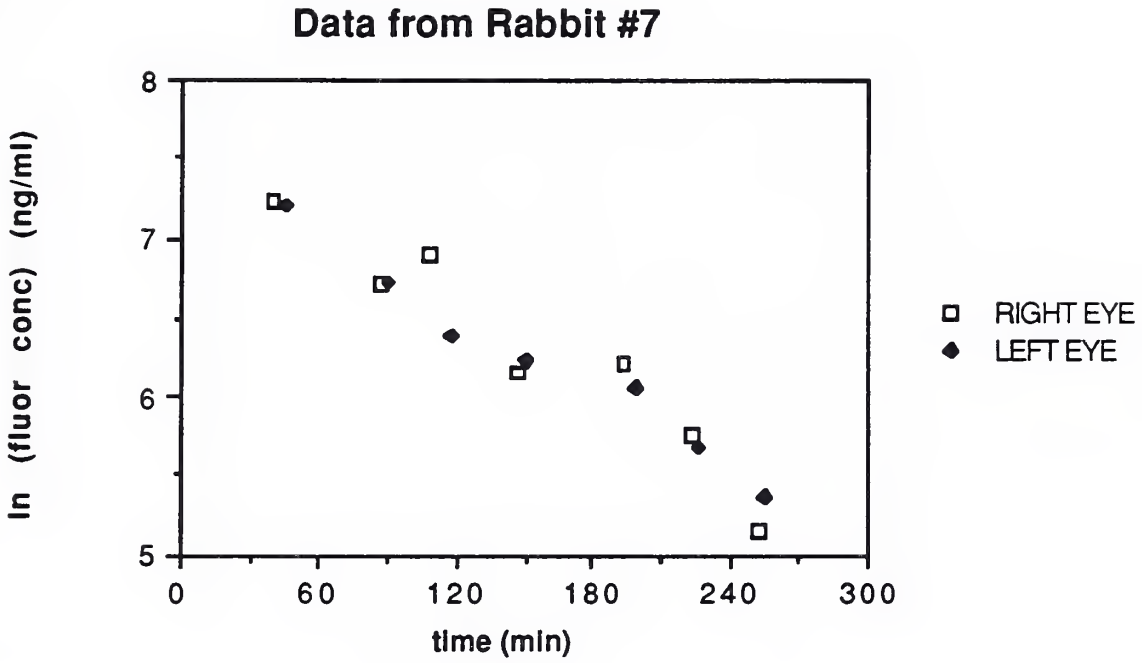


Figure 8: Aqueous humor flow determination for Rabbit #7. Scanning was begun 2 hours 25 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE	LEFT EYE
Flow (ul/min)	2.23	2.03
Slope	-8.92×10^{-3}	-8.12×10^{-3}
Corr. coeff.	0.96	0.987

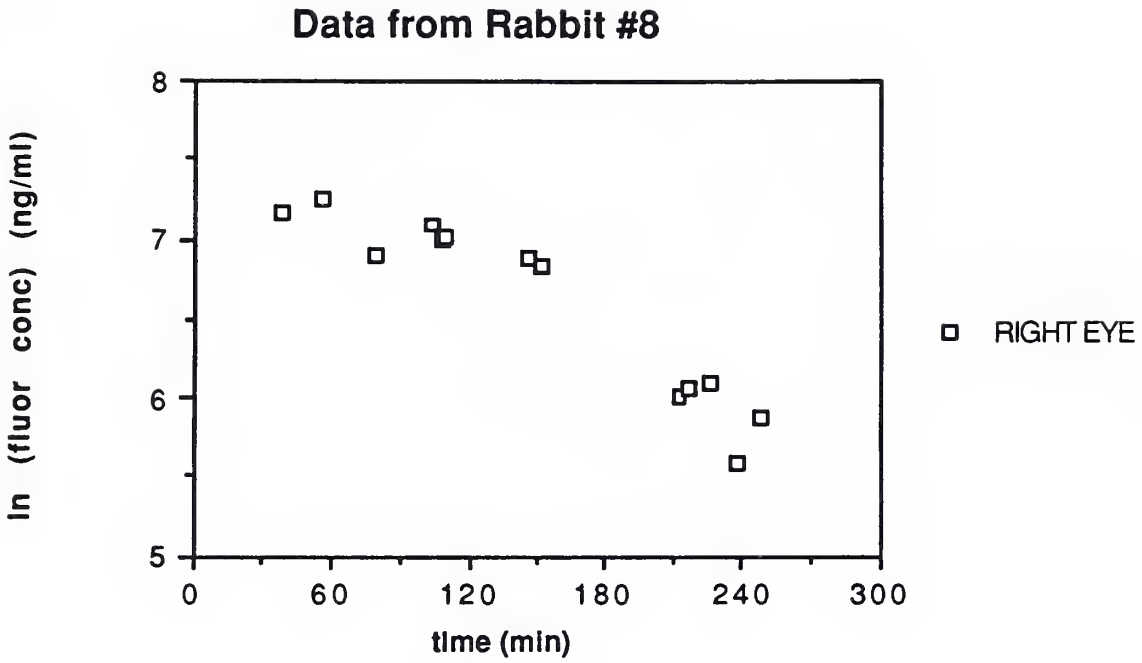


Figure 9: Aqueous humor flow determination for Rabbit #8. Scanning was begun 3 hours 30 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE
Flow (ul/min)	1.89
Slope	-7.57×10^{-3}
Corr. coeff.	0.94

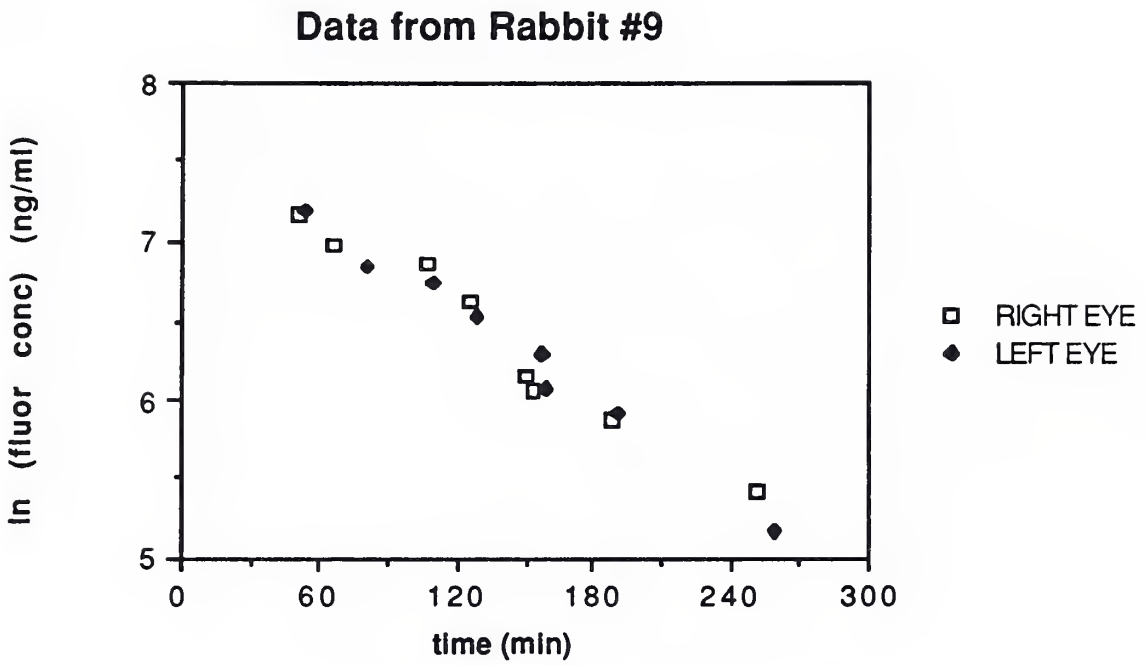


Figure 10: Aqueous humor flow determination for Rabbit #9. Scanning was begun at 2 hours 30 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE	LEFT EYE
Flow (ul/min)	2.36	2.43
Slope	-9.44×10^{-3}	-9.72×10^{-3}
Corr. coeff.	0.98	0.991

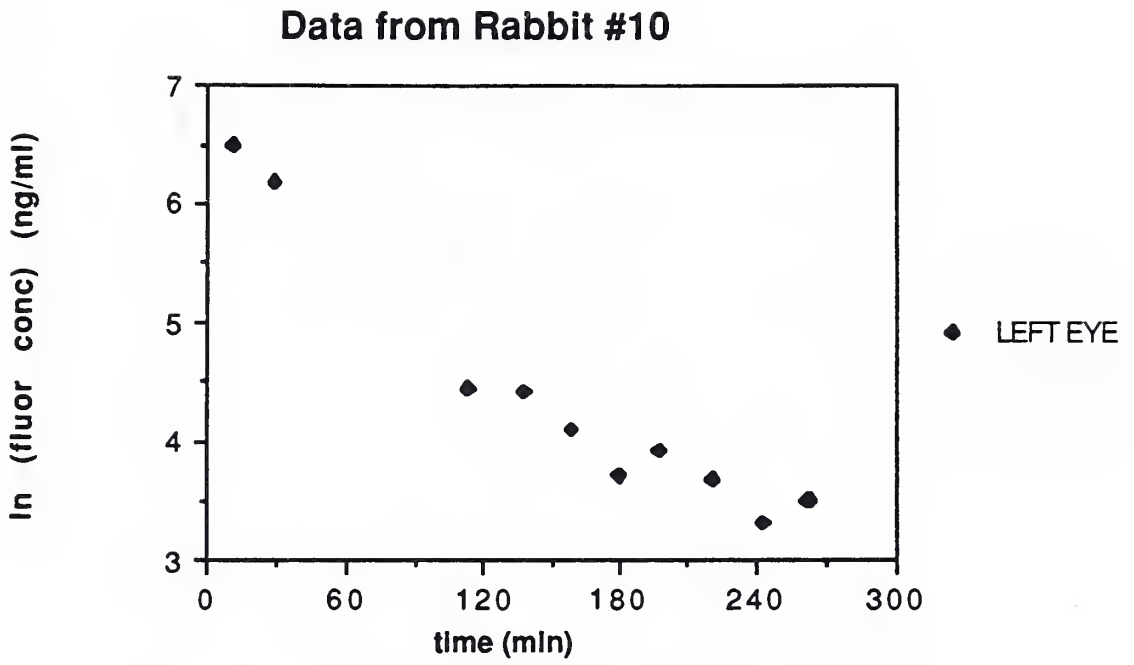


Figure 11: Aqueous humor flow determination for Rabbit #10. Scanning was begun 2 hours 20 minutes after 10 ul intracameral injection of fluorescein dextran.

	LEFT EYE
Flow (ul/min)	3.05
Slope	-0.0122
Corr. coeff.	0.96

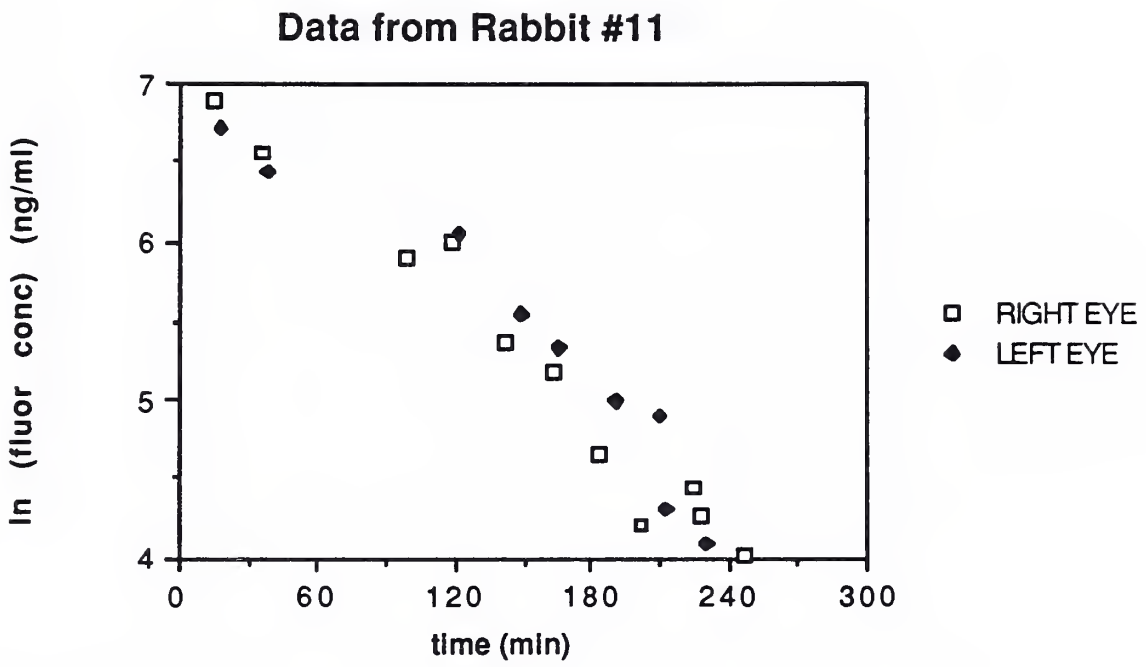


Figure 12: Aqueous humor flow determination for Rabbit #11. Scanning was begun 2 hours 0 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE	LEFT EYE
Flow (ul/min)	3.21	2.88
Slope	-0.0128	-0.0115
Corr. coeff.	0.98	0.96

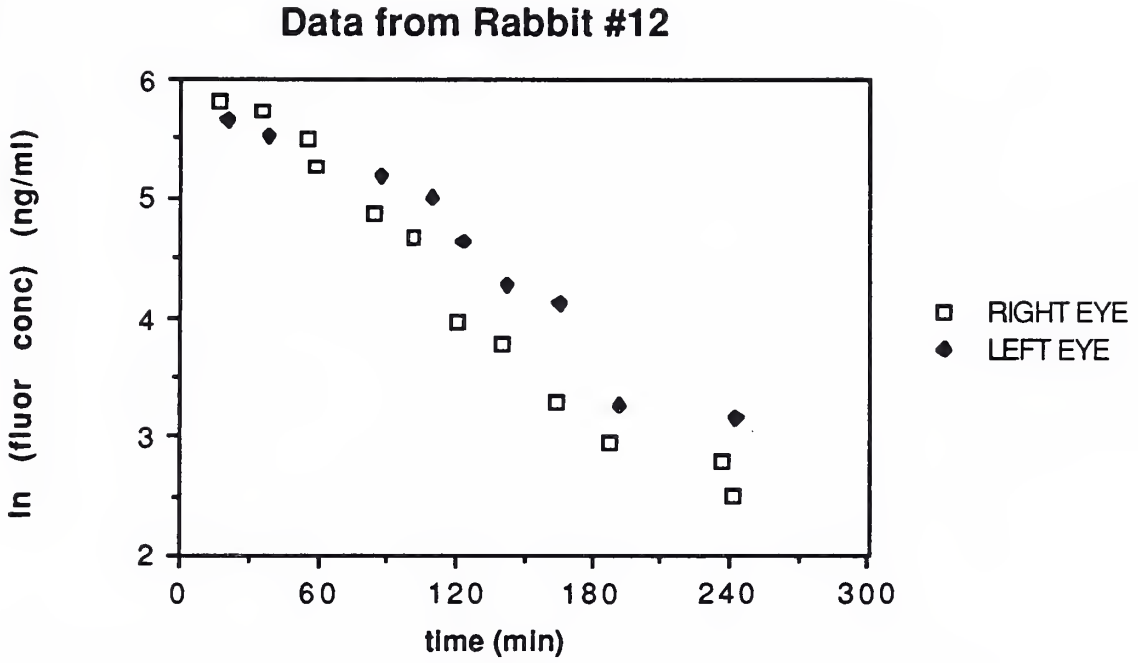


Figure 13: Aqueous humor flow determination for Rabbit #12. Scanning was begun 2 hours 45 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE	LEFT EYE
Flow (ul/min)	4.08	3.21
Slope	-0.0163	-0.0128
Corr. coeff.	0.98	0.97

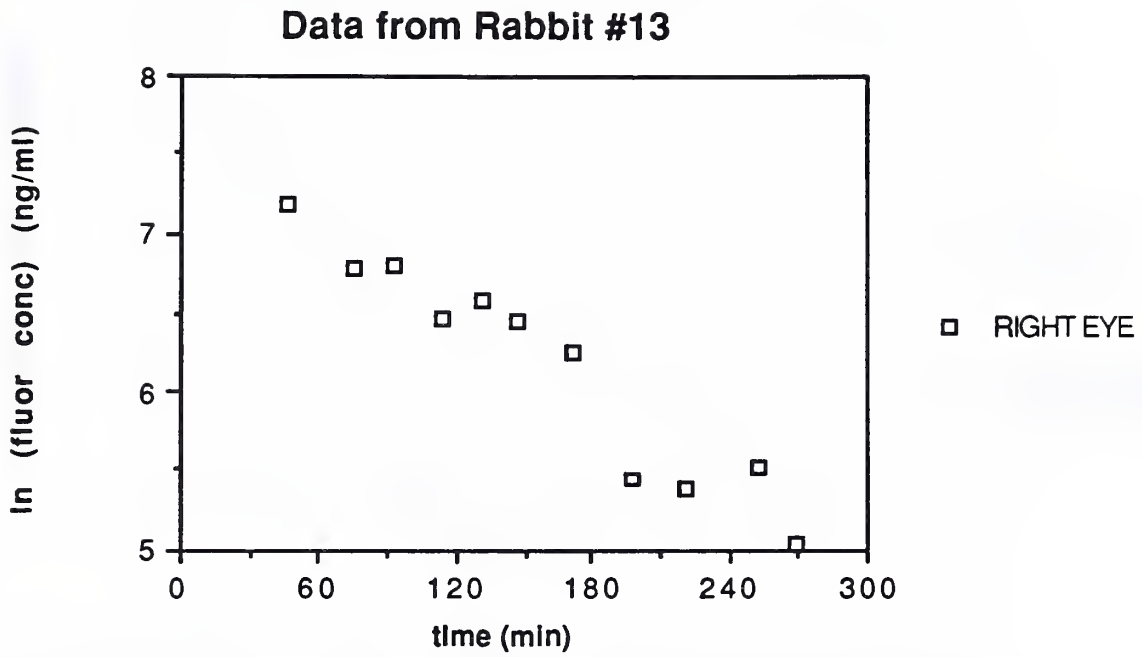


Figure 14: Aqueous humor flow determination for Rabbit #13. Scanning was begun 3 hours 0 minutes after 10 ul intracameral injection of fluorescein dextran.

	RIGHT EYE
Flow (ul/min)	2.35
Slope	-9.41×10^{-3}
Corr. coeff.	0.96

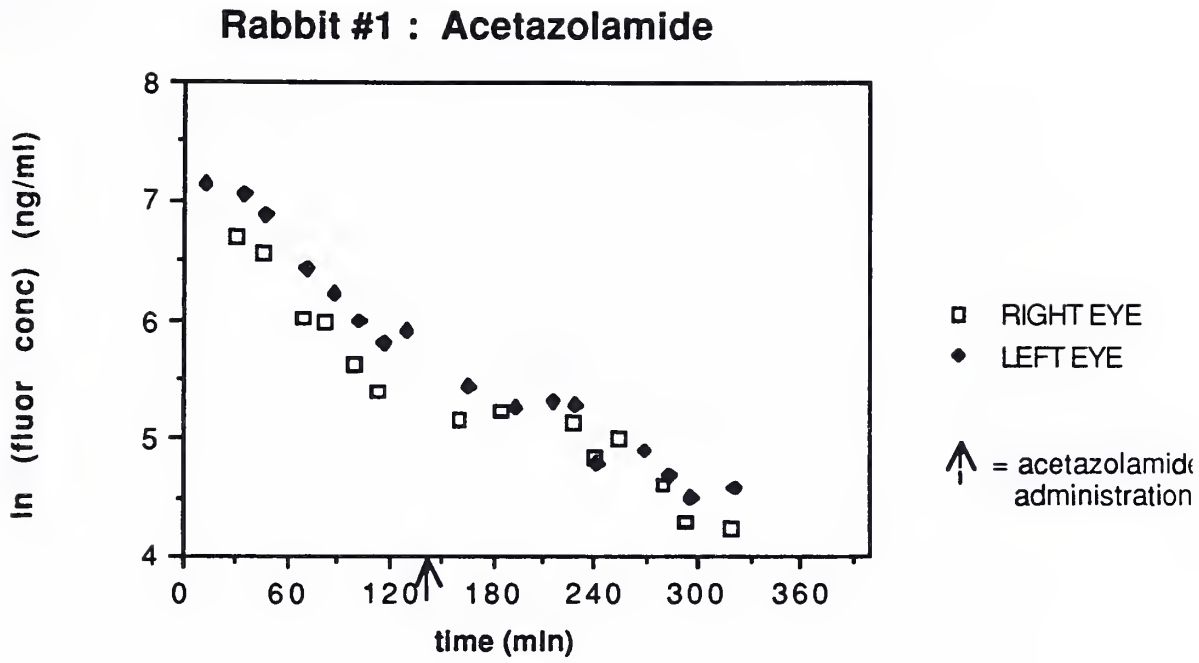


Figure 15: Determination of change in aqueous humor flow rate after intravenous administration of acetazolamide (25 mg/kg) 2 hours 30 minutes after scanning was begun. Flow rates were calculated for the 2 hours 30 minutes prior to acetazolamide administration, and for the period 1 to 3 hours following the administration.

	RIGHT EYE	LEFT EYE
Before acetazol.		
Flow (ul/min)	4.02	3.19
Slope	-0.016	-0.13
Corr. coeff.	0.994	0.98
After acetazol.		
Flow (ul/min)	2.52	1.90
Slope	-0.01	-0.0076
Corr. coeff.	0.94	0.89
Change in flow	-37%	-40%

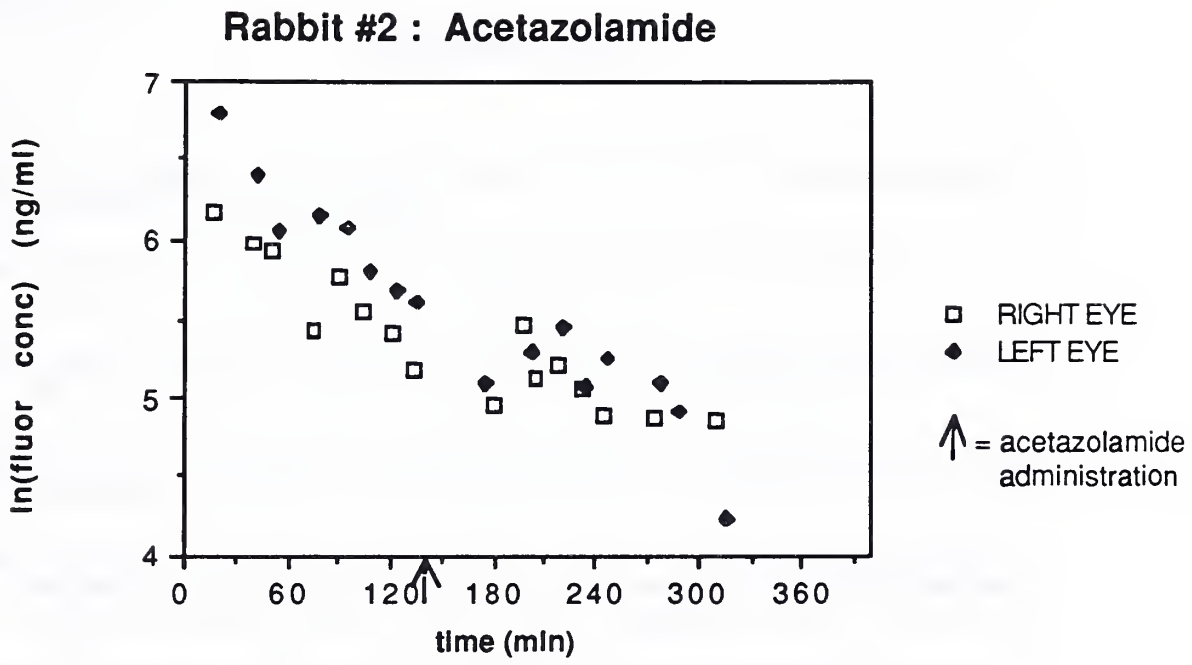


Figure 16: Determination of change in aqueous flow rate after intravenous administration of acetazolamide (25 mg/kg) 2 hours 30 minutes after scanning was begun. Flow rates were calculated for the period 2 hours 30 minutes prior to acetazolamide administration, and for the period 1 to 3 hours following the administration.

	RIGHT EYE	LEFT EYE
Before acetazol.		
Flow (ul/min)	1.94	2.27
Slope	-7.75×10^{-3}	-9.08×10^{-3}
Corr. coeff.	0.92	0.95
After acetazol.		
Flow (ul/min)	0.82	1.10
Slope	-3.26×10^{-3}	-4.41×10^{-3}
Corr. coeff.	0.84	0.79
Change in flow	-58%	-52%

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